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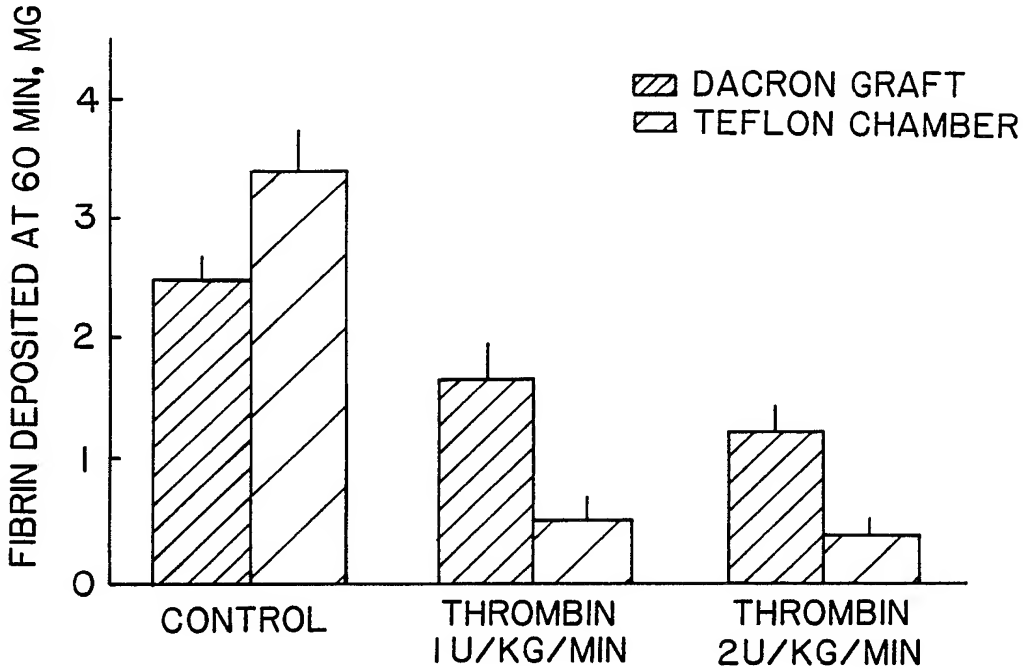


(57) Abstract

The present invention contemplates various methods of preventing and treating thrombosis via increasing circulating endogenous blood activated protein C levels and via upregulating the plasma level or generation of activated protein C. The present invention further contemplates appropriate compositions for use in inhibiting thrombosis. In addition, the present invention provides methods and compositions for determining the amount of thrombomodulin in an individual.

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# METHODS OF INHIBITING THROMBOSIS VIA ELEVATION OF CIRCULATING ENDOGENOUS ACTIVATED PROTEIN C LEVELS

## TECHNICAL FIELD

The present invention relates to the use of agents such as thrombin that increase circulating level of activated protein C (APC) to inhibit arterial, venous, and microvascular thrombosis and thromboembolism.

## BACKGROUND OF THE INVENTION

Adverse effects of the most potent antithrombotic agents at therapeutically efficacious doses limit their use in patients and make these drugs unsuitable for the prevention of thrombosis and embolism in apparently healthy individuals. For example, in diseases complicated with arterial thrombosis, the use of antithrombotic and/or thrombolytic therapy has undesired side effects, such as bleeding or reocclusion during thrombolytic treatment in myocardial infarction, bleeding or thrombosis following surgery, and thrombosis following surgery that employs grafts or other cardiovascular prosthetic devices. Also, with the possible exception of aspirin and ticlopidine, there is no evidence that any of the antithrombotic agents could provide very significant long-term prevention against coronary, cerebral or venous thrombosis. Since thrombotic occlusion of blood vessels is a causal or contributing factor in over 40% of the deaths is the United States, any agent that inhibits or prevents intravascular coagulation is of great importance.

The optimal antithrombotic therapy would have anticoagulant, antiplatelet and fibrinolytic properties simultaneously, without the attendant hazards of hemorrhage. Thus, one of our primary goals was to see whether elevation of circulating endogenous

activated protein C (APC) levels in the blood, produced by the administration of an appropriate agent, would exhibit antithrombotic properties in a reproducible, quantitative, standardized non-human primate model of thrombosis.

It has now been discovered that pharmacologic elevation of circulating levels of endogenous APC interrupts thrombosis. A standardized quantitative nonhuman primate model of arterial and venous thrombosis was used to measure the antithrombotic effect of endogenous APC, as described herein.

A thrombus is an aggregate of elements formed in the living heart or vessels from constituents of the blood in response to a thrombogenic stimulus. Thrombosis, the process of thrombus formation, can occur through distinct but usually interactive mechanisms. Platelet aggregation occurs as a result of platelets being activated by a thrombogenic stimulus such as a vessel wall lesion. Fibrin formation is the result of activation of the coagulation cascade system whose final step is typically considered the conversion of fibrinogen into fibrin by thrombin, i.e., fibrin formation. The purpose of fibrin formation in thrombi and hemostatic plugs appears, in part, to be stabilization of the aggregated platelets and stabilization of the thrombus or plug.

The magnitude or degree of participation of platelet aggregation and fibrin formation is now known to vary as a result of hemodynamic (blood flow) factors. For instance, venous thrombosis occurs under low flow rate conditions and has been shown to involve a combined and equivalent consumption of platelets and elements of the coagulation cascade system. Harker et al., N. Eng. J. Med. 287: 999-1005 (1972). As a

result, venous thrombi are typically relatively unstructured masses composed of aggregated platelets, interspersed fibrin and red blood cells; hence the name "red thrombus". See Freiman, in Hemostasis and Thrombosis: Basic Principles and Clinical Practice, 2d ed., Coleman, et al. (eds.), Philadelphia, J. B. Lippincott Co., pp. 1123-35 (1987). These observations suggest that fibrin formation plays the predominant role in venous thrombus development.

In contrast, arterial thrombosis occurs under high flow rate conditions and has been shown to involve, at least in its early stages, selective consumption of platelets. Harker et al., N. Eng. J. Med. 287: 999-1005 (1972). For example, animal studies have shown that the prosthetic surface of plastic arteriovenous cannulas produce thrombi that are composed predominantly of platelets, the thrombogenic process proceeding without significant fibrin formation. Evans et al., J. Exp. Med. 128: 877-894 (1968), and Harker et al., J. Clin. Invest. 64: 559-569 (1979). Presumably, fibrin formation is not dominant in arterial thrombosis because procoagulant material, such as thrombin, fibrin monomers, or small fibrin aggregates, is swept away from the thrombogenic focus by the rapid arterial flow before coagulation becomes fully activated and before fibrin polymerizes. As a result, arterial thrombi are typically either composed predominantly of platelets (a "white thrombus") or are a complex structure composed of a basal or primary mass of platelets and a secondary mass of platelets and fibrin that overlays, and extends downstream from, the primary mass. See Freiman, *supra*. In either case, platelet aggregation at the site of a lesion is the predominant mechanism of arterial thrombus development. Therefore, arterial

thrombosis, at least in its early stages, can be characterized as being platelet dependent. Harker et al., in Vascular Diseases: Current Research and Clinical Application, Strandess et al., (eds.) Orlando, Grune & Stratton, pp. 271-283 (1987).

In view of the foregoing, it is not surprising that the therapeutic effectiveness of agents that affect either platelet aggregation or fibrin formation has been found to depend on the type of thrombosis being treated. For instance, agents such as aspirin or ticlopidine that inhibit platelet function, i.e., inhibit the ability of platelets to aggregate, are effective in preventing arterial thrombosis but are not effective in treating stasis-type venous thrombosis. Conversely, agents such as heparin that inhibit the ability of thrombin to form fibrin have been shown to be therapeutically effective against stasis-type venous thrombosis but not arterial thrombosis. Harker et al., Thromb. Diath. Haemorrh. 31: 188-203 (1974). The art also teaches that the emphasis in effective management of arterial thrombosis should be on regulating platelet aggregation as opposed to fibrin formation. That is, therapeutically effective inhibition of platelet dependent arterial thrombosis can be based on administration of agents that inhibit the ability of platelets to adhere or aggregate (platelet function inhibitors) as opposed to agents that only inhibit fibrin formation per se (anticoagulants). Ideally, a clinically useful platelet-modifying drug should be nontoxic, have sustained action and have good antithrombotic potency without excessive risk of abnormal bleeding. None of the currently available clinical agents satisfies all these requirements. Aspirin, sulfinpyrazone, dipyridamole, suloctidil and

ticlopidine are agents that have been evaluated in clinical trials to date.

In early studies, authors described a "systemic anticoagulant activity" which they observed after infusing thrombin into healthy dogs. [See, e.g., Comp, et al., J. Clin. Invest. 70: 127-34 (1982); Quick, Am. J. Physiol. 197: 791-4 (1959); Girolami, Throb. Diath. Haemorrh. 16(1): 243-56 (1966).] *Ex vivo* testing of the canine plasma following thrombin infusion led Comp, et al. to postulate that administration of low levels of thrombin led to generation of APC, which in turn inhibited blood coagulation, and that APC initiated fibrinolysis via elevating circulating plasminogen activator levels. Comp, et al., *supra*. Colucci, et al., later reported that thrombin infusion in endotoxin-treated rabbits reduces the levels of plasminogen activator inhibitor in plasma, and they postulated that protein C (PC) might be implicated as a mediator of that mechanism. Blood 74 (6): 1976-82 (1989).

None of the above studies have proceeded in the direction taken herein, however, and it now appears that incorrect conclusions have been drawn in those earlier studies. For example, Colucci, et al. (*supra*, 1989) showed that APC infusion does not elevate plasminogen activator in monkeys. Moreover, it was not observed until recently that increasing the levels of APC via the administration of exogenous APC has an inhibitory effect on thrombosis, particularly arterial thrombosis. Even more significantly, until the present disclosure, it was not appreciated that an individual's own, **endogenous** APC pool could be elevated and effectively used to inhibit thrombosis. Further, it was not appreciated until now that thrombin, a potent inducer of thrombosis via its

clotting of fibrinogen and its activation of platelets, could be administered at low doses over a prolonged period of time in the presence of a thrombus or a thrombogenic stimulus to achieve a significant antithrombotic effect. Even more surprisingly, thrombin functions as an effective antithrombotic agent and a potent stimulator of endogenous APC levels, particularly in primates, including man.

Use of agents that stimulate increased levels of endogenous APC is clearly a practical alternative to other modes of antithrombotic therapy. For example, the current cost of 1.0 mg highly purified human α-thrombin is about $60.00. The market value of APC is unknown at present; however, the current cost of human plasma derived PC zymogen is about $1,000 per mg (Enzyme Research Laboratories, South Bend, IN). This indicates that the utilization of the endogenous PC pool for APC generation is a much more economic alternative than infusion of the active antithrombotic enzyme, APC, itself.

We have now demonstrated that increasing the level of circulating endogenous APC is a novel method for preventing and treating thrombosis. Our results indicate that thrombin, the ultimate prothrombotic factor, unexpectedly and ironically, is an antithrombotic enzyme when infused at low doses.

Therefore, agents that are capable of upregulating the plasma level or generation of APC can be used for the treatment or prevention of thrombosis and thromboembolism. Such agents include thrombin, active site acylated-thrombin, or thrombin-like enzymes; soluble thrombin:thrombomodulin complex or its analogs; agents that would prevent clearance or decay of thrombin:thrombomodulin complexes; peptides that selectively interfere with inhibition of APC;

factor Xa or its analogs; plasmin or trypsin analogs; venoms (e.g., protac, Russel Viper venom [RVV]); recombinant or plasma-derived PC or its analogs, agents that enhance PC synthesis or thrombomodulin concentration; specific antibodies against PC inhibitors; and agents that delay clearance of PC zymogen, to name a few examples.

## BRIEF SUMMARY OF THE INVENTION

It has now been found that utilization of the endogenous activated protein C (APC) pathway is an effective new therapeutic modality.

Therefore, the present invention contemplates various methods for treating and inhibiting thrombosis and/or thromboembolism formation in a patient. One such method comprises administering to the patient an effective amount of an agent capable of increasing the blood activated protein C level in the patient. In one embodiment, the agent comprises a serine protease capable of activating protein C via enzymatic cleavage. An example of such a protease is thrombin, including human thrombin. In other variations, the thrombin is selected from a group comprising plasma-derived or recombinant α-thrombin, thrombin E192Q, thrombin K52E, other naturally-occurring or mutated thrombins, and active site acylated-thrombin. Other appropriate agents capable of increasing the activated protein C level include thrombin analogs, Protac (a snake venom PC activator), factor Xa, venoms, or any enzyme -- natural or synthetic -- that is capable of activating protein C.

In various embodiments, the effective amount of agent administered is that sufficient to inhibit arterial and/or venous thromboembolic events. In other embodiments, the agent is administered intravenously, via bolus injection or via continuous

infusion, for as long a period as is efficacious.

The present invention further contemplates that an effective amount of thrombin, and more particularly α-thrombin, is administered in the dosage range of about 0.05U/kg/min (approximately 17 ng/kg/min) to about 2U/kg/min (approximately 0.666μg/kg/min). Further, the contemplated effective amount of thrombin is sufficient to achieve an APC concentration in the blood 3-5 times the normal level of APC, or more. In another embodiment, the effective amount of thrombin is sufficient to achieve an APC concentration in the blood of at least about 10 ng/ml. In another variation, the effective amount of thrombin is sufficient to maintain the activated protein C concentration for a time period of at least about 30 minutes. The invention further contemplates that an effective amount of thrombin is sufficient to achieve an activated protein C concentration in the blood of from 10 to 760 ng/ml.

In another variation, the amount of thrombin is administered over a period of at least 20 minutes. Alternatively, the effective amount of thrombin is sufficient to achieve an activated protein C concentration in the blood three to five times the normal level of activated protein C, or higher.

Yet another variation contemplates the administration of an antithrombotic agent in addition to an agent capable of increasing the activated protein C level in the blood. The antithrombotic agent may be selected from the group comprising fibrinolytic agents, oral anticoagulants, dextrans, pentoxifilline, snake venom, soluble thrombomodulin, antiplatelet drugs, cyclooxygenase inhibitors, cAMP modulators, ticlopidine, thrombolytic agents, streptokinase, Eminase, urokinase, tissue plasminogen

activator, anticoagulant peptides, protein C, and activated protein C.

The present invention also contemplates a therapeutic method in which blood of a patient circulates through a prosthetic device, which method comprises administering an effective amount of an agent to an individual that elevates the endogenous APC level substantially concurrently with exposing the blood to a prosthetic surface on the device. As contemplated herein, "substantially concurrently" is defined as occurring within about 48 hours of performing a medical or surgical procedure on a patient. In a preferred embodiment, the therapeutic method further comprises a method of inhibiting thrombosis or thrombus formation in a prosthetic device, which method comprises administering an effective amount of an agent to the patient substantially concurrently with exposing the blood to a prosthetic surface on the device, wherein the agent is capable of increasing the blood activated protein C level in the patient. The prosthetic device may be selected from the following group, for example: (a) cardiopulmonary assist devices, (b) hemodialysis devices, (c) an arterial prostheses, (d) venous prostheses, (e) arteriovenous shunts, (f) metallic or polymeric endoprostheses; (g)heart valve implants or artificial heart valves, and (h) an artificial heart.

In another variation, the present invention contemplates a method of treating surgically induced thrombosis, which method comprises administering an effective amount of an agent to a patient substantially concurrently with performing an invasive surgical procedure on the patient, wherein the agent is capable of increasing the blood activated protein C level in the patient.

The invention further discloses a method of performing invasive surgery on a patient, which method comprises administering an effective amount of an agent to the patient, the administration being performed during a time period of up to one hour prior to exposing the circulating blood of the patient to a prosthetic surface on a cardiopulmonary assist device, wherein the agent is capable of increasing the blood activated protein C level in the patient. In other embodiments, the agent is administered during or subsequent to the invasive surgical procedure, or both.

In another variation, the invention contemplates a method of performing a medical procedure on a patient, which method comprises administering an effective amount of an agent to the patient, the administration being performed during a time period of up to one hour prior to initiating the medical procedure, wherein the agent is capable of increasing the blood activated protein C level in the patient. In other embodiments, the invention further comprises continuing to administer the effective amount of agent during or subsequent to the performance of the medical procedure, or both. Examples of contemplated medical procedures include angioplasty, hemodialysis, treatment of heparin-induced thrombosis, use of endoprosthetic devices (e.g. stents), use of intravascular access devices or indwelling endovascular devices (e.g. cannulas, catheters, and devices used with injections and infusions), or thrombolytic therapy.

In other embodiments, the present invention contemplates methods for determining the presence of thrombomodulin, particularly that which is active or functional, in an individual, comprising the steps of

(a) obtaining a first plasma sample from the individual (which sample may be collected in one or more vials, tubes, or other appropriate vessels, or which may be divided into separate portions for testing); (b) determining the protein C level in a first portion of the sample; (c) determining the activated protein C level in a second portion of the sample; (d) intravenously injecting a thrombomodulin-dependent protein C activating agent into a vein of the individual for 5 minutes; (e) obtaining a second plasma sample from the individual (i.e., a sample taken subsequently to the intravenous injection of protein C activating agent; (f) determining the activated protein C level in the second sample; and (g) comparing the activated protein C levels determined for the first and second samples to determine the presence of functional thrombomodulin in the individual. In various embodiments, the protein C activating agent comprises thrombin, which may be administered at a rate of 0.1U/kg/min or more.

The present invention also contemplates therapeutic compositions suitable for administration via injection, comprising an effective amount of an agent capable of increasing the blood activated protein C level via enzymatic cleavage of protein C in a pharmaceutically acceptable carrier or excipient, wherein the effective amount of the agent is administered at the dosage of 0.05U/kg/min to 2U/kg/min. In other variations, the agent is present in a solution of up to 0.5U agent per ml of carrier; it may also be infused over a period of at least 20 minutes. In one embodiment, the agent is thrombin; in other embodiments, the agent may comprise plasma-derived or recombinant α-thrombin, thrombin E192Q, thrombin K52E, other naturally-occurring or mutated

thrombins, active site acylated-thrombin, thrombin analogs, Protac (a snake venom PC activator), factor Xa, venoms, or any enzyme --natural or synthetic -- that is capable of activating protein C. It is further contemplated that active site acylated thrombin may be administered in conjunction with an anticoagulant specific for thrombin. In various embodiments, acyl-thrombin may be co-administered with one or more of such anticoagulants, or in a "cocktail" form with one/or more such anticoagulants including peptides such as polypeptides derived from prothrombin, or "PT polypeptides".

The present invention further contemplates various diagnostic compositions comprising an effective amount of a thrombomodulin-dependent protein C activating agent in a pharmaceutically acceptable carrier or excipient, wherein the agent is administered at a suggested dosage of 0.1U/kg/min to 2U/kg/min. In various embodiments, the agent may be present in a solution of up to 0.5U agent per ml of carrier and may be infused over a period of at least 5 minutes. In one embodiment, the agent is thrombin.

The present invention also contemplates the *ex vivo* activation of protein C via use of an external shunt apparatus in conjunction with an apparatus containing a thrombin:thrombomodulin complex, followed by reintroduction of APC into the individual. The present invention also contemplates a method of decreasing the rate of clearance of endogenous APC in order to elevate the concentration of circulating APC. For example, administration of inhibitors of PCI, PAI-I, α-1-antitrypsin, and α-2-antiplasmin is suggested.

Yet another embodiment contemplates the inhibition of thrombosis or thromboembolism via increasing endogenous zymogen levels and/or APC

levels, via administration of anabolic steroids (e.g. danazolol), for example. Another aspect of the present invention contemplates the administration of thrombin to an individual as a means of assaying for the presence of thrombomodulin *in vivo*.

BRIEF DESCRIPTION OF THE DRAWINGS

In the drawings forming a portion of this disclosure:

Figure 1 illustrates a thrombogenic device in an AV shunt such as that used in the present study of arterial and venous thrombosis. It illustrates the direction (from the Dacron graft end through the Teflon chamber portion) and speed (100 ml/min) of blood flow, as well as the approximate dimensions of the graft portions (20mm in length and 4 mm i.d. for the Dacron portion; 20 mm in length and 9.3 mm i.d. for the Teflon portion). Examples of construction materials and gamma camera range are also indicated.

Figure 2 illustrates the generation of circulating activated protein C during infusion of thrombin. Activated protein C (APC) in nanograms (ng)/ml is plotted against time, in minutes. Mean data from the control animals (open triangles) is compared against that from animals that are receiving thrombin infusions in 1U/kg/min (open squares) and 2U/kg/min (open circles) doses. In all animals, a thrombogenic device is present.

Figure 3 illustrates platelet deposition in the dacron graft segment in control animals and in experimental animals, during infusion of thrombin. Platelets deposited (in billions) are plotted against time (in minutes). In all instances, a thrombogenic device is present. Experimental animals are receiving thrombin infusions in 1U/kg/min (open squares) and 2U/kg/min (open circles) amounts. Control data is

illustrated using open triangles.

Figure 4 illustrates platelet deposition in the teflon chamber portion of the grafts in control animals (open triangles) and in experimental animals, during infusion of thrombin. Platelets deposited (in billions) are plotted against time (in minutes). In all instances, a thrombogenic device is present. Experimental animals are receiving thrombin infusions in 1U/kg/min (open squares) and 2U/kg/min (open circles) amounts.

Figure 5 illustrates the deposition of fibrin in thrombogenic devices during infusion of thrombin. Fibrin deposition (in milligrams, mg) is measured in control animals and in those to which thrombin (T) has been administered in doses of 1U/kg/min and 2U/kg/min, respectively. Deposition of fibrin in the dacron portion of the graft and in the teflon chamber portion is compared to the controls.

Figure 6 illustrates the prolongation of clotting time during infusion of thrombin. Activated partial thromboplastin time (APTT, measured in seconds) is plotted against time (in minutes) for control (open triangles) and test animals. In all instances, a thrombogenic device is present. Experimental animals are receiving thrombin infusions in 1U/kg/min (open squares) and 2U/kg/min (open circles) amounts.

Figure 7 illustrates the inhibition of thrombus formation during *in vivo* protein C activation by thrombin. Inhibition of platelet (PLT) and fibrin (FN) deposition, expressed as a percentage, is plotted against thrombin infusion, in U/kg/min. Inhibition in the dacron and teflon chamber graft segments is illustrated. Data for platelet deposition in the dacron portion is illustrated using open triangles; that for platelets in the teflon chamber portion is

shown using an X-shaped mark. Fibrin deposition in the dacron portion is illustrated with open circles; that for fibrin in the teflon chamber is shown using open squares.

Figure 8 illustrates the inhibition of thrombus formation during *in vivo* protein C activation by thrombin. Percent inhibition of platelet (PLT) and fibrin (FN) deposition is plotted against percent increase in activated protein C (APC), in thousands. Inhibition in the dacron and teflon chamber graft segments is illustrated. Data for platelet deposition in the dacron portion is illustrated using open triangles; that for platelets in the teflon chamber portion is shown using an X-shaped mark. Fibrin deposition in the dacron portion is illustrated with open circles; that for fibrin in the teflon chamber is shown using open squares.

Figure 9 illustrates the correlation between APC levels and PC activity in 67 blood donors (each donor is indicated by a square). In the vertical axis, APC activity in percent (%) is shown and is plotted against PC activity in percent (%), which is diagrammed on the horizontal axis. As illustrated, r has a value of 0.61 and p has a value of <0.001.

## DETAILED DESCRIPTION OF THE INVENTION

### A. Definitions

"Activated protein C" (APC) refers to a member of the serine protease family subgroup involved in the blood coagulation pathway. Protein C (PC) is a zymogen, that is, it is inactive until converted into APC through interaction with thrombin, another serine protease active in the blood coagulation pathway or through interaction with other proteases. PC and APC differ in primary structure only in a dodecapeptide which is present at the amino-terminal end of PC and

absent in APC. The 12 amino acid peptide is removed by proteolytic cleavage. The role of APC is to inactivate coagulation cofactors Va and VIIIa.

"Anticoagulant" refers to an agent that interrupts coagulation and thereby inhibits fibrin formation.

"Coagulation" refers to the sequential process in which the multiple coagulation factors of the blood interact resulting in the formation of fibrin.

"Enzyme" as used herein refers to a protein or polypeptide capable of accelerating or producing by catalytic action some change in a substrate for which it is often specific.

"Prosthetic device" refers to a biologic or synthetic vascular prosthesis that is inserted into the vasculature so as to receive and/or transport blood.

"Protease" as used herein refers to a protein that catalyzes the cleavage of peptide bonds in other proteins.

"Serine protease" as used herein refers to a member of a family of proteases that share an active site functional domain defined by amino acid residues $Asp^{102}$, $Ser^{195}$, and $His^{57}$ of chymotrypsin. Examples of serine proteases include those in the complement convertase family (e.g., factors Clr, Cls, D, C3 convertase); those in the plasminogen activator family (e.g., plasmin, tissue plasminogen activator (tPA), urinary plasminogen activator (uPA)); those in the blood coagulation pathway family (e.g., factors XIIa, XIa, Xa, IXa, VIIa, thrombin, plasma kallikrein, APC), those in the digestive enzyme family (e.g., trypsin, chymotrypsin, pancreatic elastase, enterokinase), those in the hormone processing family (e.g., tissue kallikreins, post proline cleaving enzyme), and the

like. "Serine protease", as used herein, is further intended to encompass all substantially homologous molecules.

"Substantially homologous" means that a particular subject sequence or molecule, for example, a mutant sequence, varies from a reference sequence by one or more substitutions, deletions, or additions, the net effect of which does not result in an adverse functional dissimilarity between reference and subject sequences. For purposes of the present invention, amino acid sequences having greater than 90 percent similarity, equivalent biological activity, and equivalent expression characteristics are considered substantially homologous and are included within the scope of proteins defined by the terms "serine protease" and "thrombin". Amino acid sequences having greater than 40 percent similarity are considered substantially similar. For purposes of determining homology or similarity, truncation or internal deletions of the reference sequence should be disregarded, as should subsequent modifications of the molecule, e.g., glycosylation. Sequences having lesser degrees of homology and comparable bioactivity are considered equivalents.

"Thrombin", as used in the present application, is a multifunctional serine protease. As a procoagulant enzyme, thrombin clots fibrinogen, activates clotting factors V, VIII, and XIII, and activates platelets. It is also capable of cleaving the dodecapeptide which is present at the amino-terminal end of PC and absent in APC; the 12 amino acid peptide is removed by proteolytic cleavage. "Thrombin", as used herein, is further intended to encompass all substantially homologous molecules, including the mutated thrombins known as E192Q and

K52E. [See Le Bonniec, PNAS USA 88: 7371-7375 (1991), and Wu, et al., PNAS USA 88: 6775-6779 (1991).] One unit (U) of thrombin, as used herein, is generally known in the art, and means equivalent fibrinogen clotting activity to 1 N.I.H. unit of reference enzyme using the same assay (see Fenton, et al., Thromb. Res. 4: 809-817 (1974). Thrombin may be administered according to the present application in a pharmaceutically acceptable form.

B. Introduction

Activated protein C (APC) is an anticoagulant enzyme that exhibits potent antithrombotic effects when given at pharmacologic doses in venous, microvascular and arterial thrombosis models. (See, e.g., Taylor, et al., J. Clin. Invest. 79: 918-925 (1987); Emerick, et al., "Preclinical pharmacology of activated protein C," in The Pharmacology and Toxicology of Proteins, A.R. Liss, ed., pp. 351-367 (1987); Gruber, et al., Blood 73: 639-642 (1989); Gruber, et al., Circulation 82: 578-585 (1990); Araki, et al., Thromb. Res. 62: 209-216 (1991).) APC inhibits the blood coagulation pathways and the formation of thrombin at least in part by proteolytic cleavage of coagulation cofactors Va and VIIIa, and APC also enhances fibrinolysis, at least *in vitro*. (See, e.g., Clouse and Comp, NEJM 314: 1298-1304 (1986); Esmon, Science 235: 1348-1352 (1987).)

APC can be generated from its vitamin K-dependent zymogen, protein C (PC), upon enzymatic activation by thrombin, thrombomodulin-bound thrombin, factor Xa bound to thrombomodulin, trypsin or various venoms, including snake venoms, *in vitro*. (See, e.g., Stenflo, J. Biol. Chem. 251: 355-363 (1976); Kisiel, J. Cl. Invest. 64: 761-9 (1979); Escom and Owen, PNAS USA 78: 2249-2252 (1981); Haley, et al., J. Biol.

Chem. 264: 16303-10 (1989).) Infusion of low doses of thrombin into normal, healthy dogs prolonged the *in vitro* clotting time of the plasma prepared from the blood during thrombin infusion. These experiments proposed that thrombin-induced activation of the protein C pathway might occur *in vivo* [Comp, et al., J. Clin.Invest. 70: 127-34 (1982)], but no antithrombotic effect in either venous or arterial thrombosis was suggested or demonstrated. Moreover, Cowp, et al. did not contemplate the use of thrombin itself as an antithrombotic agent. These experiments also failed to answer the question whether the PC system is a physiologic regulator of venous or arterial thrombosis. Subsequent studies indicated that inherited and acquired deficiency in PC was associated with thromboembolytic disease [see, e.g., Griffin, et al., J. Clin. Invest. 68: 1370-73 (1981); Gruber, et al., Throm. Res. 42: 579-581 (1986); Taylor, et al., J. Clin. Invest 79: 918-925 (1987); Kurosawa-Ohsawa, et al., Blood 75: 2156-2163 (1990)], which suggests an important regulatory role for the PC pathway.

APC is unique among known anticoagulants, since it circulates in blood, and it has now been shown to inhibit many kinds of thrombosis (arterial, microvascular, and venous) at pharmacologic doses that do not compromise primary hemostasis. (See, e.g., Gruber, et al., *supra* (1989); Emerick, et al., *supra* (1987); Taylor, et al., *supra* (1987).) However, antithrombotic therapy with purified plasma-derived APC or recombinant APC requires infusion of considerable quantities (>0.07 mg/kg/h) of enzyme to reach therapeutically efficacious plasma levels of the active enzyme in arterial-type thrombosis. (See Gruber, et al., Circulation 84(6): 2454-2462 (1991).)

This makes the therapeutic use of APC expensive. Further, the parenteral route of administration requires close supervision by medical personnel.

APC is a member of the serine protease family subgroup involved in the blood coagulation pathway. Protein C (PC) is a zymogen, that is, it is inactive until converted into APC through interaction with thrombin, another serine protease active in the blood coagulation pathway. PC and APC are structurally different only in a dodecapeptide which is present in PC and absent in APC. The 12 amino acid peptide is removed by proteolytic cleavage, and such proteolytic cleavage permits the acquisition of enzymatic activity by APC. One major role of APC is to inactivate coagulation cofactors Va and VIIIa. Therefore, APC can down-regulate thrombosis through its activity on factors Va and VIIIa. APC is inactivated by the protease inhibitors, α-1-anti-trypsin, plasminogen activator inhibitor-1, α-2-antiplasmin, α-2-macroglobulin, and possibly by other nonspecific proteases.

The level of APC and/or PC in a body fluid sample has medical relevance. For instance, the incidence of hereditary PC and protein S deficiency among thrombophilic patients [Gladson et al., Thromb. Haemost. 59: 18-22 (1988)] is higher than in the normal population [Miletich et al., N. Engl. J. Med. 317: 991-996 (1987)] and many patients have been described with heterozygous PC deficiency and familial thrombophilia [Griffin et al., J. Clin. Invest. 68: 1370-1373 (1981); Horellou et al., Br. Med. J. 289: 1285-1287 (1984); Bovill et al., Blood 73: 712-717 (1989)]. Complete deficiency of PC activity, whether inherited [Branson et al., Lancet 2: 1165-1168 (1983); Seligsohn et al., N. Engl. J. Med. 310: 559-562

(1984)], experimental [Taylor et al., J. Clin. Invest. 79: 918-925 (1987); Snow et al., Circulation 82: 711-769 (1990)], or acquired [Gruber et al., Thromb. Res. 42: 579-581 (1986); Mitchell et al., N. Engl. J. Med. 317: 1638-1642 (1987)], represents a potentially fatal condition. We have discovered that the normal level of circulating APC in an individual is about 2 to 3 ng/ml; thus, a therapeutic method which is capable of increasing endogenous APC levels is clinically important.

Thrombotic complications of PC deficiency can be controlled with PC or APC replacement therapy (Seligsohn et al., Taylor et al., and Snow et al., *supra*) or liver transplantation [Casella et al., Lancet 1: 435-437 (1988)]. The presence of measurable quantities of APC-inhibitor complexes in plasma samples from patients with intravascular coagulation indicates that APC is generated *in vivo* [Heeb et al., Blood 73: 455-461 (1989); Tabernero et al., Thromb. Haemost. 63: 380-382 (1990)].

We now hypothesize that utilization of the body's own antithrombotic pathway may be an ideal modality for both treatment and prevention of thrombosis. Our calculations based on the published concentration of the zymogen in plasma indicate that the circulating pool of the proenzyme (PC zymogen) averages 11 mg in a normal adult of 60kg. [See, e.g., Griffin, et al., J. Clin. Invest. 68: 1370-73 (1981).] Calculating with a half-life of 7 hours for PC [Seligsohn, et al., NEJM 310: 559-562 (1984)], the protein is synthesized and released into the bloodstream at a rate of 0.8 mg/hour under resting conditions, when no excessive consumption occurs. This reserve would allow a source for continuous generation of active endogenous enzyme with sufficient antithrombotic efficacy, comparable to

that of the exogenous purified APC preparations.

To test our hypothesis, we chose to infuse thrombin, a natural activator of the PC zymogen, reasoning that thrombin, by combining with its endothelial cofactor, thrombomodulin (TM), would generate active circulating APC enzyme in non-human primates with experimental thrombosis. We then hoped to observe whether induced APC at levels significantly higher than normal concentrations in plasma would potently inhibit both venous-type and arterial-type thrombus formation in thrombogenic devices and the vascular tree.

The method of inhibiting thrombosis disclosed herein, which utilizes the body's own, endogenous protein C pathway, is an entirely new therapeutic modality. Its clinical significance is comparable to the introduction of plasminogen activators (streptokinase, urokinase, tissue-type plasminogen activator, streptokinase-plasminogen complexes, etc.) in the treatment of thrombotic vascular occlusions, since those approaches also utilize an endogenous antithrombotic pathway, the fibrinolytic pathway of the body. Some of our earlier studies provided evidence that **exogenous**, infused APC is a potent antithrombotic enzyme at plasma levels higher than 100 ng/ml. Unexpectedly, our data shows that increasing the **endogenous** APC levels by infusing a protein C activator -- i.e., stimulating the body's own reserves, as opposed to infusing APC directly -- results in potent antithrombotic effect as well.

C. Therapeutic Applications

1. Prosthetic Devices

Arterial prosthetic devices having surfaces exposed to arterial blood when operatively inserted into a patient's circulation are well known in the

art. See, Biologic and Synthetic Vascular Prostheses, J. Stanley, ed., Grune and Stratton, N.Y. (1982). Exemplary biological arterial prostheses include, without limitation, autologous arterial grafts, particularly autologous saphenous vein arterial grafts, dialdehyde starch-tanned bovine heterografts, human umbilical vein grafts and the like. Synthetic arterial prostheses are also well known in the art and include Dacron grafts, expanded polytetrafluoroethylene grafts such as those described in U.S. Patent No. 3,962,153, hydrophobic polymer-lined grafts such as those described in the U.S. Patent No. 4,687,482, and the like.

Exemplary arterial prosthetic surfaces include arterial stents, A-V shunts, and the like. A-V shunts are typically sections of non-endothelialized tubing, usually constructed of a polymeric material, that are used to transport arterial blood in a vein, either directly or first through and *ex vivo* therapeutic device. Exemplary *ex vivo* therapeutic devices include hemodialysis and cardiopulmonary assist devices, and the like. The use of *ex vivo* therapeutic devices is well known in the art.

A thrombo-resistant vascular prosthesis of the present invention is produced by a method comprising removably affixing an endogenous APC-level-increasing agent onto the luminal surface of the prosthesis. Thus, removably affixing such an agent to the luminal surface of a vascular prosthetic device is a method for improving the thrombo-resistance of that device.

2. Therapeutic Methods

We propose that therapy using agents that increase circulating endogenous APC, alone or in combination with other antithrombotic agents, will be useful in various clinical settings, including the

following, without limitation:

a. Acute arterial thrombotic occlusion including coronary, cerebral or peripheral arteries.

b. Acute thrombotic occlusion and restenosis after angioplasty. Angioplasty, a commonly performed procedure in cardiology and vascular medicine, disrupts intimal surfaces of diseased arteries and may cause acute occluding thrombosis. It also may frequently produce restenosis several months later, due to platelet-mediated intimal thickening. These complications represent a major unsolved problem in cardiology. Induction of endogenous APC alone or in combination with other antithrombotic agents may be an effective means of intervention.

c. Reocclusion after thrombolytic therapy. Thrombolytic agents such as tissue plasminogen activator (tPA), streptokinase, or Eminase salvage ischemic tissue when used within hours of acute heart attack or stroke by re-establishing blood flow in the occluded artery. At present, between one-fourth and one-third of patients who have successful thrombolytic re-perfusion of occluded coronary arteries subsequently undergo reocclusion after discontinuing thrombolytic agent infusion. This complication occurs despite full-dose heparin therapy. Endogenous APC should have greater efficacy than heparin in preventing reocclusion because it inhibits arterial thrombosis.

d. Small caliber vascular graft occlusion. Vascular grafts of small caliber, e.g., 3mm diameter, have a high frequency of thrombotic occlusion. The experimental data are directly relevant to the possibility that endogenous APC alone or in combination with other antithrombotic agents could be a useful agent to prevent occlusion.

e. Endogenous APC could be used in other instances of arterial thrombosis or thromboembolism where other applied therapeutic measures (e.g. heparin, aspirin, thrombolytic agents, etc.) are either contraindicated or ineffective. The advantage provided by the safety of the use of exogenous APC indicate that endogenous APC could improve the results in the treatment of the following conditions, for example: acute pre- or post-capillary occlusion, including transplantations, retinal thrombosis, heparin-induced thrombosis, microthrombotic necrosis of any organ, complicating infections, or tumors, thus possibly reducing the medical and social costs. Since combined administration of APC with urokinase has additive antithrombotic effects [Gruber, et al., *supra* (1991)], combined administration of agents that increase endogenous APC and thrombolytic agents (e.g., tPA, urokinase, prourokinase, streptokinase, Eminase, or acylated forms of plasmin or plasminogen, will likely reduce the dose of thrombolytic agent needed and the number of hazardous and expensive complications of thrombolytic therapy.

f. Hemodialysis. The prosthetic surfaces and flow design of all hemodialyzers are thrombogenic. Currently, heparin is infused during dialysis. However, heparin is only partially effective, thereby limiting the reuse of dialyzers. In addition, heparin has a number of troublesome side effects and complications. Endogenous APC could replace heparin or complement its beneficial effects.

g. Cardiopulmonary bypass surgery. To prevent thrombus formation in the oxygenator and pump apparatus, heparin is currently used. However, it fails to protect against platelet activation and the resultant transient platelet dysfunction which

predisposes bleeding problems post-operatively. The antithrombotic effects of endogenous APC in the absence of compromised surgical hemostasis is an important aspect of the presently disclosed strategy for the use of APC induction with surgical procedures.

h. Left ventricular cardiac assist device. This prosthetic pump is highly thrombogenic and results in life threatening thromboembolic events -- complications that are only partially reduced by use of conventional anticoagulants (e.g., heparin or coumarin-type drugs).

i. Heart replacement -- use of artificial heart. This prosthetic device has four artificial valves and biomer chamber construction, which are all highly thrombogenic. Although heparin is partially effective, it fails to prevent platelet-mediated thromboembolic events. Artificial hearts could have much greater utility if the risk of thromboembolism could be reduced by using the body's own ability to produce circulating APC.

j. Deep vein thrombosis and pulmonary embolism. Thrombotic occlusion of the major veins and subsequent embolism are commonly occurring conditions with relatively high acute mortality. Although other anticoagulants and fibrinolytic agents are partly effective, they do not substantially reduce the mortality of pulmonary embolism. Postthrombotic syndrome still occurs, even after and during conventional antithrombotic therapy. Induction of the PC pathway proximal to the thrombosis, i.e., on the endothelial microvascular surface, could reduce the damage caused by venous occlusion by releasing the powerful endogenous anticoagulant right at the site it is needed.

k. Microvascular thrombosis. Thrombotic

occlusion of the precapillary, capillary and postcapillary system results in irreversible ischemic tissue necrosis unless the formation of the microthrombi are prevented or the thrombi are dissolved. Tissue necrosis, besides the loss of function of the necrotized tissue, can lead to generalized toxic conditions often leading to fatality. Disseminated intravascular coagulation can occur without complete occlusion of microvessels but with substantial fibrin deposition in the microvessel, resulting in failure of normal gas and electrolyte exchange and metabolism. Fibrin deposition and thrombotic occlusion of microvessels may occur as a result of infections, hemolysis, antigen-antibody reactions, adult respiratory distress syndrome, amniotic fluid embolism during labor and delivery, and several other conditions. Traditional antithrombotic agents (e.g.,heparin, fibrinolytic agents, oral anticoagulants) often fail to protect from the disastrous consequences of microthrombosis. Infused purified APC has been shown to be efficacious and to prevent fatality in toxic microthrombosis followed by experimental *E. coli* infection. Thus, an increase in the circulating levels of endogenous APC could provide antithrombotic protection in microvascular thrombosis. As discussed in the Examples below, infusion of 0.2 mg thrombin (1 U/kg/min) throughout one hour generated APC plasma levels of about 160-359 ng/ml. Similar antithrombotic plasma levels were achieved by infusion of 2.5 mg purified exogenous APC for one hour in previous experiments. (See Gruber, et al., *supra* (1989).)

3. Agents Which May Be Combined to Produce an Increase in Endogenous APC Levels

A number of agents may be administered according

to the present invention, in order to achieve the desired increase in endogenous blood APC levels. For example, thrombin may be administered in combination with APC, protein C, fibrinolytic agents, and oral anticoagulants, to name a few examples.

Various agents which may be used to increase endogenous APC levels and/other agents useful in a combined therapy with agents that increase circulating APC are set forth in Table 1 below. It should be noted that some enzyme inhibitors including hirudin, PPACK, heparin, heparinoids, SP54, dermatan sulfate, and the like are not recommended for use in combination with thrombin. They may, however, be administered in combination with other agents that increase circulating APC, including protein C and APC itself.

TABLE 1

| Mechanism | Target | Agent |
|---|---|---|
| Block enzyme specificity heparin, sulfate | Coagulation factors (IIa, Xa, IXa, XIa) | Enzyme inhibitors: hirudin, PPACK, heparinoids, SP54, tick peptide, dermatan |
| Alter enzyme activity | IIa, Xa | Soluble thrombomodulin |
| Deplete zymogens | Coagulation factors (X, IX, VII, II) | Oral anticoagulants |
| Modify surface | Platelets | Antiplatelet drugs, cyclooxygenase inhibitors (aspirin, NSAD), cAMP modulators (dipyridamole, $PGI_2$ and analogs), ticlopidine |
| | Blood vessels | Vascular grafts/ angioplasty |
| Remove thrombus | Fibrin | Thrombolytic agents (streptokinase, Eminase, tcu-PA, scu-PA, t-PA), bat fibrinolysin, surgery |
| Neutralize cofactors | FVa, FVIIIa | Activated protein C |
| Modify viscosity/ flow | Plasma | Dextrans |
| Remove fibrinogen | Fibrinogen | Snake venoms (Ancrod, reptilase) |
| Modify erythrocyte rheology | RBC | Pentoxifilline, ticlopidine |

D. Therapeutic or Diagnostic Compositions

The preparation of therapeutic compositions which contain antithrombotic or anticoagulant agents as active ingredients is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions, however, solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified. The active ingredient is often mixed with inorganic and/or organic excipients which are pharmaceutically acceptable and compatible with the active ingredient (e.g., a protein C-activating or APC-increasing agent). Preferred excipients are protein carriers, such as those used in insulin formulations. Suitable excipients may include, for example, water, saline, dextrose, glycerol, or the like, and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, or pH buffering agents which enhance the effectiveness of the active ingredient.

The composition is conventionally administered intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic or diagnostic composition used in the present invention refers to physically discrete units suitable as unitary dosages for humans, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic or diagnostic effect in association with the required excipient.

The composition is administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be

administered depends on the subject to be treated, capacity of the subject's blood antithrombotic system to utilize the active ingredient, the degree of antithrombotic effect desired. Precise amount of active ingredient required to be administered depends on the judgement of the practitioner and are peculiar to each individual. However, suitable therapeutic dosage ranges for thrombin, and particularly human α-thrombin, are of the order of 0.05U to 2U per kilogram body weight per minute and depend on the route of administration. Suitable diagnostic dosage ranges for thrombin are of the order of 0.05U to 2U per kilogram body weight per minute and depend on the route of administration, as well. Administration of thrombin in a solution of about 0.5U/ml or less is suggested.

Pharmaceutically acceptable, biodegradable excipients suitable for use in slow release formulations are well known and include polymers (e.g., polyethylene glycol), polyamino acids (e.g., polyglycolic acid), and the like. When a slow release formulation containing an agent that increases the endogenous APC level is removably affixed to the luminal surface, the local concentration of an agent in the blood (i.e., the concentration of the agent that increases the APC level at the blood-luminal surface interface) will be proportional to the excipient dissolution rate. While the ratio of agent to excipient depends, as is well known in the art, on the choice of excipient and blood flow conditions through the prosthetic flow channel, therapeutically effective amounts of an endogenous APC-level-increasing agent can be delivered to blood contacting the luminal surface for a period of from hours to days when a slow release formulation is used.

Alternatively, thrombin may be administered in a

bolus -- i.e., a more concentrated mass -- as opposed to steady infusion. Reversible acylation of the active center of serine proteases, e.g. trypsin, plasmin and thrombin, has been shown to modify the biochemical properties of the enzymes. Following acylation, enzyme activity is lost and the enzyme activity is inhibited as long as the acyl group is bound to the enzyme. However, there follows a slow generation of enzyme activity due to a slow deacylation reaction. This slow deacylation effectively allows a controlled release of active enzyme from a pool of inactive acylated enzyme. (See, e.g., Chase, et al., Biochemistry 8(5): 2212-2224 (1969).) For example, acylation of the active center of the streptokinase-plasminogen complex led to the development of a new thrombolytic agent known as APSAC (anisolated plasminogen streptokinase activator complex -- which has also been referred to as Eminase or Anistreplase), that exerts its effect by slow deacylation in the circulation. Eminase is currently used clinically for thrombolysis. Thus, it is contemplated that active site acylated-thrombin can be used as a bolus injection rather than a slow, continuous infusion in patients with thrombosis, to activate protein C. (See, e.g., Smith, et al., Nature 290: 505 (1981).) Inactivation of the active center of thrombin by a small molecular inhibitor, e.g., by diisopropylfluorophosphate, does not affect its binding to its endothelial receptor, TM. However, small molecular weight active site inhibitors do ablate thrombin's interaction with its substrates, e.g., fibrinogen, coagulation factors and cofactors (for example, Factors V and VIII), and antithrombins. Thus, injected acyl-thrombin would preferably bind to its high affinity receptors such as TM. At the time

of deacylation, the thrombin bound to TM molecules will predominantly activate the circulating PC zymogen or react with antithrombin but will not cleave fibrinogen or activate platelets or factors V and VIII. Thus, acyl-thrombin may be used via bolus injection for the purpose of increasing endogenous circulating APC levels with equivalent -- or superior -- efficacy compared to continuous infusion of thrombin, and will provide a wider therapeutic window. Acylated forms of other enzymes (e.g., factor Xa) which increase the endogenous level of APC may be used under similar conditions and considerations. Acyl-thrombin would preferably be administered in a dosage sufficient to achieve essentially a "controlled release" in the 0.05U-2U/kg/min range suggested for infused thrombin.

Because acyl-thrombin does not cleave fibrinogen, activate platelets or activate factors V and VIII, it is ideal not only for solo administration, but can function efficaciously when administered in conjunction with an anticoagulant specific for thrombin. For example, acyl-thrombin may be co-administered with one or more of such anticoagulants, or in a "cocktail" form with one/or more such anticoagulants.

Examples of appropriate anticoagulants specific for thrombin include, but are not limited to, peptides such as polypeptides derived from prothrombin, or "PT polypeptides". A PT polypeptide inhibits the activity of its corresponding serine protease, prothrombin, and has anticoagulant activity. A serine protease binding domain sequence is an amino acid residue sequence that defines a surface-exposed region of a native and naturally folded serine protease protein, a region that participates in an essential protein-protein

interaction between the serine protease and another protein during the catalytic process of the serine protease. By "essential protein-protein interaction" is meant that if the interaction is perturbed by a competition reaction using a PT polypeptide, the catalytic activity of the serine protease is diminished or extinguished. Thus, the interaction is essential for the protease to exert its catalytic activity.

Copending U.S. Patent Application Serial No. 07/793,989 to Griffin and Mesters, filed November 18, 1991 and entitled "Serine Protease-Derived Polypeptides and Anti-Peptide Antibodies, Systems and Therapeutic Methods for Inhibiting Coagulation", identifies examples of appropriate anticoagulants specific for thrombin, i.e., PT polypeptides. In particular, peptides with exosites corresponding to those identified by sequence ID No. 4 and residue number 557-571 (PT polypeptide 4:557-571) may be appropriately used in combination with acylated thrombin. The term "exosite" indicates a region of a serine protease that is defined by amino acid residues which are not located at the region of the protease responsible for the catalytic activity; i.e., an exosite is not located at the catalytic site. Other anticoagulant peptides may also be administered in conjunction with acyl-thrombin.

E. Diagnostic Applications

According to present knowledge, thrombin exerts its PC activating effect via binding to the endothelial thrombin receptor, thrombomodulin (TM). (See, e.g., Esmon, et al., PNAS USA 78: 2249-52 (1981).) Deficiency in TM, i.e., an insufficient number of these cellular receptors for thrombin, either acquired or inherited, could lead to a

pathological condition characterized by a deleterious distribution of thrombin molecules between thrombomodulin and procoagulant receptors. For example, this would result in higher available thrombin levels for fibrinogen and for platelet receptors, resulting in fibrin formation and platelet aggregation (i.e., thrombosis). Furthermore, this would result in a decrease in endogenous APC generation that separately in and of itself would result in increased risk for thrombosis. Deficiency of TM has not been described to date, since no available assay has been available to detect this condition, until now.

When exogenous thrombin is infused, the newly-formed thrombin:thrombomodulin complexes generate circulating APC from protein C. In cases of TM deficiency, this response to thrombin infusion will be decreased. Thus, assaying the APC increase after thrombin infusion provides a method of detecting TM functional deficiency in individuals. The term "functional thrombomodulin" is used herein to refer to active, functioning TM, i.e., TM having the ability to complex with thrombin and to generate APC from circulating PC zymogen.

An assay for TM may be formed essentially as follows. First, the functional PC levels of the individual to be tested for TM deficiency should be assayed and found normal; then, if necessary, they should be normalized via exogenous PC infusion. Second, blood should be drawn from the individual and assayed, using the APC ECA or some other appropriate method, to determine the baseline APC level. Third, slow intravenous injection of dilute human thrombin (or other agents that activate PC) for 5 minutes at a suggested rate of 0.1U/kg/min in a solution of

≤0.5U/ml will be made to increase APC levels. The APC increase will be impaired in thrombomodulin-deficient individuals. Finally, the enzyme capture assay (ECA) for APC, or some other method for determining APC levels in blood drawn from a vein, artery or capillary bed should be performed. The results of infusing thrombin or other agents that increase APC should be analyzed as an increase in APC from the baseline value in percentage terms, as well as in absolute concentration and compared to reference values for normal, healthy subjects. It is anticipated that the increase of circulating APC levels in response to thrombin administration will be impaired in TM-deficient individuals.

The present invention also describes a diagnostic system, preferably in kit form, for assaying for the presence of protease in a fluid sample. A diagnostic system includes, in an amount sufficient for at least one assay, a subject protease activity-free immobilized anti-protease antibody as a separately packaged immunochemical reagent. Instructions for use of the packaged reagent are also typically included.

As used herein, the term "package" refers to a solid matrix or material such as glass, plastic, paper, foil and the like capable of holding within fixed limits a protease activity-free anti-protease antibody of the present invention in an immobilized composition. Thus, for example, a package can be a microtiter plate well to which microgram quantities of a contemplated antibody have been operatively affixed, i.e., linked so as to be capable of being immunologically bound by an antigen, and being protease-free. Preferred are anti-APC antibodies, and particularly MabC3 in an immobilized composition.

"Instructions for use" typically include a

tangible expression describing the reagent concentration or at least one assay method parameter such as the relative amounts of reagent and sample to be admixed, maintenance time periods for reagent/sample admixtures, temperature, buffer conditions and the like. The packaging materials discussed herein in relation to diagnostic systems are those customarily utilized in diagnostic systems.

F. Experimental Apparatus

The vascular prosthesis of the present invention, in its preferred embodiment, is substantially non-compliant. As used herein the expression "non-compliant" means showing less than 10 percent expansion of the inner diameter between systole and diastole under normal arterial pressures (less than 250 mmHg). The external surface of the vascular prosthesis permits tissue anchoring upon implantation in a human or other mammals, as is common for currently commercially available prostheses.

The tubular segment of the vascular prosthesis may be constructed of materials that exhibit the requisite strength, durability and suturability. Commercially available materials suitable for use in fabricating the prosthesis or graft include a polyester such as Dacron (C. R. Bard, Inc., Billerica, MA) and a polyfluorocarbon such as Teflon (Gore-Tex, W. L. Gore, Flagstaff, AZ).

In preferred embodiments the luminal surface is comprised of polymers that form a relatively smooth, non-polar and hydrophobic surface. Such materials and either use in forming a portion of a luminal surface as described in U.S. Patent No. 4,687,482 to Hanson, whose disclosures are incorporated herein by reference.

The following examples are intended to

illustrate, but not limit, the present invention.

EXAMPLES

1. Thrombosis Model

To study and describe the rate of thrombus formation *in vivo* in a manner free of uncontrolled variables, a primate model of vascular graft thrombosis was used. Baboons were chosen for these studies because they appear to have thrombotic processes similar to humans.

Normal male juvenile baboons (*Papio anubis*) had permanent thrombogenic exteriorized silicone rubber arteriovenous access shunt (3mm interior diameter [i.d.]) surgically placed between the femoral artery and vein. An illustration of a model of such a device is set forth in Figure 1.

Briefly, a chronic arteriovenous shunt was surgically implanted between the femoral artery vein (A-V shunt) of a normal, 10-12 kilogram male baboon. The permanent shunt system consisted of two 25 centimeter (cm) lengths of silastic tubing having an inner diameter (i.d.) of 3 millimeters (mm; Dow Corning Corp., Midland, MI) connected to 13- and 15-gauge Teflon vessel tips (Lifemed, Venitron Corp., Compton, CA). In addition, the two Silastic lengths were fixed with Dacron sewing cuffs (E.I. duPont de Nemours and Co., Wilmington, DE) at skin exit sites. Blood flow was established by connecting the two Silastic shunt segments with a 1 cm length of blunt-edge Teflon tubing (2.8 mm i.d.). In various protocols described below, thrombogenic graft devices were subsequently operatively linked to the animal by interposing them between the segments of the permanent Silastic A-V shunt.

Thrombus formation was initiated by incorporation of a thrombogenic device into the shunt. Figure 1 is

illustrative of the device described. The thrombogenic segment consisted of a Dacron vascular graft segment (2cm long, 4mm i.d.) exhibiting high-shear undisturbed blood flow, similar to arterial flow. Distal to the Dacron graft was placed a Teflon-coated chamber (2cm long, 9.3mm i.d.) exhibiting low-shear annular disturbed blood flow and stasis, which more closely mimics venous flow. The procedures and the model have been extensively described and tested for reproduceability. (See, e.g., Hanson, et al., Thromb. Haemost. 53: 423-27 (1985); Hanson, et al., Thromb. Haemost. 58: 801-05 (1987); Gruber, et al., Blood 73: 639-642 (1989); and Gruber, et al., Circulation 82: 578-585 (1990); see also Cadroy, et al., J. Clin. Invest. 84: 939 (1989); Cadroy, et al., J. Lab Clin. Med. 113: 436 (1989); Cadroy, et al., PNAS USA 88: 1177-1181 (1991); and Kelly, et al., Blood 77: 1006-1012 (1991).)

Blood passed into the arterial end of the device at a flow rate of about 100 ml/min. Turbulence generated in the venous portion of the device was observed to simulate that of an actual vein.

Both graft- and chamber-induced acute thrombosis was indicated by continuous deposition of platelets and fibrinogen from the circulation onto the thrombogenic surfaces. This device generated two types of thrombi during perfusion. The proximal Dacron segment accumulated platelet-rich arterial-type thrombi, while the distal chamber accumulated erythrocyte and fibrin-rich thrombi. [See also Cadroy, et al., J. Clin. Invest. 84: 939 (1989) and Cadroy, et al., J. Lab Clin. Med. 113: 436 (1989) (1989)].

The blood flow in the shunt and device was detected using a Doppler flow meter. Progressive

thrombotic occlusion of the grafts occurred after 70 minutes of blood flow through the shunt. The number of deposited platelets and the quantity of fibrin deposited was used as a quantitative marker of thrombus formation. Platelet deposition was measured in real time using radiolabeled autologous platelets and gamma-camera imaging, according to the methods of Hanson, et al., Arteriosclerosis 5: 595-603 (1985) and Cadroy, et al., PNAS USA 88: 1177-1181 (1991). Fibrin deposition was quantified as described in Gruber, et al., *supra* (1990) or Cadroy, et al., *supra* (1991). The thrombogenic device was incorporated into the shunt at 0 minutes, and was removed after 60 minutes of blood perfusion through the shunt; then, the normal flow was restored in the shunt.

In order to increase circulating levels of APC, we increased the concentration of thrombin:thrombomodulin complexes in the vasculature. This was achieved by infusion of thrombin (α-thrombin was a gift of Dr. John W. Fenton, Albany, NY) distal to the thrombogenic devices, into the inferior vena cava. (The α-thrombin is preferably purified according to the method described by Fenton, et al., in Chemistry and Biology of Thrombin, Lundblad, Fenton and Mann (eds.), Ann Arbor Science Publ. Inc., 1977, pp. 43-70.) Following one control experiment, thrombin was infused at two doses on different subsequent days: 1 U/kg/min and 2 U/kg/min. Calculating with an estimated 1 L/min cardiac output and negligible inhibition of the enzyme within the seconds necessary to reach the heart, these doses resulted in maximal estimated thrombin concentrations of 75 and 150 pM, respectively, in the pulmonary artery. Because of thrombin's prothrombotic properties, we also measured platelet and fibrinogen

consumption from the circulation during thrombin infusions.

2. Enzyme Capture Assay (ECA)

In order to determine the correlation between circulating endogenous APC levels and the inhibition of thrombosis, APC levels were measured in the systemic arterial blood using an enzyme capture assay (ECA) for APC.

a. Purified APC Standards

Purified APC was used as standard in the ECA. APC was prepared by isolating PC according to the following method, and then activating it. [See also Gruber, et al., Blood, *supra* (1989).]

i. Purification

Monoclonal antibodies specific for human PC light chain antibodies were further purified by immunoaffinity. C3-Mab antibodies purified from a Mono Q HR/5 column (see Geiger et al. (1989), Thromb. Haemost. 61: 86-92 and Clezardin et al. (1985), J. Chromatogr. 319: 67-77) were coupled to CNBr-activated Sepharose 4B (Pharmacia; 3 mg protein/ml gel) in coupling buffer (0.5 mol/L NaCl, 0.05 mol/L borate, pH 8.5) overnight at 4°C to form C3-sepharose. The affinity column was used to purify PC as follows. Five grams of factor IX concentrate (740 mg prothrombin complex/gr powder, vapor heated; gift from Dr. Hans Peter Schwarz, Immuno, Austria) was passed over C3-Sepharose in 250 ml buffer containing 0.1 mol/L NaCl, 2 mmol/L EDTA, 2 mmol/L benzamidine, 0.02% Na-azide, 0.02% Tween-20, 0.02 mol/L Tris-HCl, pH 7.4, and subsequently eluted with 3 mol/L NaSCN in 1.0 mol/L NaCl, 4 mmol/L benzamidine, 2 mmol/L EDTA, 0.02% Na-azide, 0.05% Tween-20, and 0.05 mol/L Tris, pH 7.0. The passage rate was 15 ml/hr at room temperature. The column was washed with 6.8 bed volumes of buffer

and eluted at a flow rate of 15 ml/hr. The PC was >95% pure when analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% gel; SDS-PAGE). Equivalent results were obtained when expired Proplex (Factor IX complex; Hyland Therapeutics, Glendale, CA) was used instead of Factor IX. 30mg of PC was then coupled to 10 ml of Sepharose. This PC-Sepharose gel was used for immunoaffinity purification of anti-PC antibodies, i.e., C3-Mab.

The purified IgG fraction from the Mono Q-HR/5 column [IgG fractions were purified using anion exchange chromatography, as described in Geiger, et al., *supra* (1989), and Clezardin et al., *supra* (1985)] then was further purified by contact with the PC-Sepharose column as follows. Purified IgG was absorbed to immobilized PC in 0.01 M Tris, pH 7.4, 0.14 M NaCl (TBS), 0.02% Na-azide, and subsequently eluted using either 3 M Na-thiocyanate in 0.05 M Tris, pH 7.4, 1.0 M NaCl, 0.02 % Na-azide or 0.1 M glycine, pH 2.5, 0.1 M NaCl. The thiocyanate eluate was dialyzed against 0.05 M Tris, pH 7.4, 0.5 M NaCl; the glycine eluate was dialyzed against TBS. Both eluates were stored at -70°C until use. The two antibody preparation methods gave equivalent results.

ii. Activation

For the ECA assay, APC was prepared as follows. The activated form of PC (APC) was prepared by treating the inactive zymogen PC prepared above with α-thrombin-Sepharose beads. Thrombin was purchased from Enzyme Research Laboratories (South Bend, IN) and prepared from homogeneous human prothrombin by activation with Factor Xa, Factor Va and phospholipid. Human thrombin was homogeneous as judged by 10% SDS-PAGE. The purified thrombin was coupled to CNBr-activated Sepharose as described. Thrombin-

Sepharose beads were mixed with purified PC solutions continuously and the activation of PC was monitored. Activation was by the method of Marlar, et al., Blood 59: 1067 (1982).

To monitor the activation of protein C, the amidolytic activity was determined in an assay using the chromogenic substrate, S-2238 (H-D-Phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide dihydrochloride, Kabi-Vitrum, Franklin, OH). Other preferred substrates include S-2366 and Spectrozyme PCa (available from American Diagnostic, Greenwich, CT). In the assay, 60 $\mu$l of the sample was admixed to 600 $\mu$l of 0.8 mM amidolytic substrate in a buffer consisting of 0.05 M Tris-HCl and 0.10 M NaCl at pH 8.0. The rate of absorbance change at a wavelength of 405 nm/minute was determined. Activation was stopped when no further increase in amidolytic activity could be detected. The resultant APC product was determined to be greater than 95% pure when analyzed by SDS-PAGE as described above.

The specific anticoagulant activity of APC was determined to be 250 Units/mg. By definition, 1U of APC corresponds to the activity of PC in 1ml of human plasma after full activation. The concentration of the APC used in the assay was initially optimized with respect to the sensitivity of the assay towards APC-induced prolongation of clotting time compared to clotting time without APC. [See Gruber, et al., Blood, *supra* (1989).]

Immunoaffinity purified APC preparations were tested for anticoagulant activity and preparations exhibiting maximal specific activity (250 U/mg) were used as standards in the APC ECA. Standard serial dilutions of purified human APC in the range of 38 ng/l to 10 mg/l were made in dilution buffer, aliquots

were frozen in liquid nitrogen and stored at - 70°C.

b. Preparation of Monoclonal Antibody C3-Mab

Murine Mabs to PC were prepared by a modification of the method of Kohler and Milstein [Nature 256: 495 (1975)]. In brief, on Day 1, BALB/c mice were injected intraperitoneally with 35 μg of purified PC mixed with complete Freund's adjuvant. On days 18 and 25 the mice were injected with 35 μg antigen in incomplete Freund's adjuvant. On day 35, three days prior to cell fusion, 35 μg of purified PC was injected intravenously (IV).

Spleen cells (8.6 X $10^8$) from immunized mice were fused with P3X63-Ag8.653 (available from ATCC as CRL15800) murine myeloma cells (1.7 X$10^8$) using 30% (wt/vol) polyethylene glycol-1000. After two days of 4 X $10^{-7}$ mol/L aminopterin treatment, the cells were seeded into 96-well microtiter plates at 1.5 X $10^4$ cells per well. Fused cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 20% newborn calf serum, 10% NCTC (National Cancer Tissue Culture) 109 medium, oxaloacetate (1 mmol/L), pyruvate (0.45 mmol/L), glutamine (2 mmol/L), penicillin and streptomycin (5ml each to 720 medium), Hepes (20 mmol/L), hypoxanthine (1 x $10^{-4}$ mol/L), and thymidine (3 X $10^{-5}$ mol/L).

Ten days post fusion, the hybridomas were screened for growth. Approximately 15% were growing. The supernatants from the growing cells were tested on day 19 for reactivity against purified PC by a direct binding radioimmunoassay (RIA). For these assays, each well of a 96-well plastic microtiter plate (Immulon II microtiter plates, Dynatech Laboratories) was coated overnight at 4°C with 6 μg/ml PC in 0.05 M borate buffer, pH 8.4. The plates were washed with 50 mM

Tris/HCl, 0.14 M NaCl, 0.05% $NaN_3$, pH 7.2, and 7 mM EDTA (TBS/EDTA), and then coated with 3% BSA in TBS/EDTA (blocking buffer) for 1 hr at 37°C. The plates were washed three times with TBS/EDTA. Fifty μl of supernatant from each clone were added to each well, along with 5 ul of 70 mM EDTA, and incubated for 1 hr at room temperature. The plate was washed four times with Buffer A/BSA + EDTA (50 mM Tris/HCl, 0.14 M NaCl, 0.05% $NaN_3$, 0.1% BSA, 1.5 mM $MgCl_2$, 0.05% Tween-20, pH 7.2, 7 mM EDTA). Iodinated rabbit anti-mouse IgG (diluted in Buffer A/BSA + EDTA) was added to each plate and it was allowed to incubate 2 hr at 37°C. Finally, the plate was washed four times with Buffer A/BSA + EDTA, the plate was dried, and the wells cut out and counted. Wells demonstrating radioactivity higher than that recorded in control plates (not coated with PC or not treated with antibody) were indicative of immunoreaction of the iodinated second antibody with anti-PC bound to immobilized PC, and thus were deemed to have been contacted with monoclonal antibody immunoreactive with PC.

Many hybridomas, including hybridoma 22A101CS3B2 (referred to herein as C3), were determined by these procedures to immunoreact with PC and were then cloned by limiting dilution. Hybridoma C3 has been deposited pursuant to Budapest Treaty requirements with the American Type Culture Collection (ATCC), Rockville, MD, on July 3, 1991, and was assigned accession number HB 10820. Hybridoma C3 was deposited in a depository affording permanence of the deposit and ready accessibility thereto by the public upon the issuance of a patent, under conditions which assure that access to the hybridoma will be available during the pending of the patent application to those deemed by the Commissioner to be entitled to such access, and that

all restrictions on the availability to the public of the hybridoma as deposited will be irrevocably removed upon the granting of the patent. The deposited hybridoma will be maintained by the ATCC for the term of the patent or 30 years from the date of deposit, whichever is longer, and in all events for at least five years after the date of the last request for access.

Thymus cells (5 X $10^5$/ml) from BALB/c mice were added to the wells as a feeder layer during cloning. The clones were evaluated in the above-described assay for production of antibodies specific for PC. Clones that were again found positive were recloned by the same procedure to ensure monoclonality and were again screened by one of the assays. Positive cell lines were selected for injection (1 X $10^6$ cells per animal) into the peritoneal cavity of BALB/c mice that had been injected with 300-500 $\mu$l pristane about 10 days prior to injection with hybridoma cells.

The ascitic fluids obtained from mice injected with clones producing monoclonal antibodies C3, C4, and C10 were screened for their ability to bind radiolabelled PC as follows. The IgG fractions were purified from the fluid using anion exchange chromatography, as described in Geiger et al. (1989), Thromb. Haemost. 61: 86-92 and Clezardin et al. (1985), J. Chromatogr. 319: 67-77. Briefly, 100 $\mu$l mouse ascites fluid in 100 $\mu$l of 20 mM carbonate, pH 9.0, was applied to a Mono Q HR 20/20 column on a Pharmacia FPLC apparatus at a flow rate of 1.0 ml/min. Elution was by a step gradient of 0% buffer B (0.05M Tris, 1 M NaCl, pH 7.4) for 5 min, 0-20% B in 30 min, and 20-35% B in 10 min.

50 ul of each purified monoclonal anti-PC antibody or nonspecific mouse IgG (62.5 ng - 4$\mu$g per

well) in 0.01 M Na-carbonate buffer, pH 9.6, were used to coat wells of Falcon 3911 Microtest II plates overnight at 4°C. The plates were washed three times with PBS, pH 7.4, containing 2% BSA, 0.05% Na-azide, 0.05% Tween 20 and three times with PBS, pH 7.4, containing 0.05% Na-azide, 0.05% Tween 20. The remaining binding sites were blocked with 200 ul of 3% BSA in PBS, 1mM EDTA and 0.05% Na-azide, 0.05% Tween 20, pH 7.4 for at least 1 hr at 37 C. $^{125}$I-PC was prepared by iodination of purified PC using the standard chloramine T method [McConahey et al. (1966) Int. Arch. All. Immunol. 29: 185-189]. Fifty ul of $^{125}$I-PC (200,000 CPM/well, specific activity 10 $\mu$Ci/$\mu$g) in 0.01 M Na-phosphate buffer containing 0.14 M NaCl (PBS), 3.0% BSA, 1 mM EDTA, 0.05% Tween 20, 0.05% Na-azide, pH 7.4, were added to each well and the plate was incubated for 90 min at 37°C. After washing the plates, wells were cut out and counted in a Micromedic 4/600 automatic gamma counter. Wells demonstrating radioactivity higher than that recorded in control please (coated with control IgG or not contacted with $^{125}$I-PC) were indicative of immunoreaction between iodinated PC and anti-PC monoclonal antibody, and thus were deemed positive.

The data indicated that all tested monoclonals immunoreacted with PC antigen. Mouse control IgG did not bind PC. As monoclonal C3 had the highest affinity for PC, it was selected for use in the APC assay described below. Murine monoclonal antibodies specific for human PC light chain antibodies were further purified by immunoaffinity, as described herein.

The wells of a 96 well flat bottom Immulon II microtiter plate (Dynatech Laboratories, Chantilly, USA) were coated with 250 $\mu$l of C3-Mab (50 to 100

μg/ml, purified as described above) in coating buffer at 4°C. The following coating buffers were used with equivalent results: 0.01 M sodium carbonate, pH 9.2, 0.02% Na-azide; or 0.02 sodium carbonate, pH 8.5, 0.02% Na-azide; or 0.02 Tris-HCl, pH 7.8, 0.02% Na-azide. Coating buffer alone was added to negative control plates. The plates were allowed to incubate for 14 hours at 4°C. The plates were then blocked with 1% casein in coating buffer (300 μl/well) for at least 1 hour at 37°C or overnight at 4°C, the latter being preferred. [The blocking buffer was stored at 4°C with immobilized soybean trypsin inhibitor (1 ml bead to 500 ml buffer; Pierce, Rockford, IL), and was filtered through 0.2 μm pore size syringe filter prior to use.] The C3-Mab coated plates were washed with washing buffer (0.02 M Tris, pH 7.4, 0.15 M NaCl, 0.02 M EDTA, 0.02% Tween 20, 0.02% Na-azide; filtered with 0.2 μm pore size filter) and stored at 4°C.

c. Preparation and Analysis of Serine Protease-Free Immobilized C3-Mab

i. Preparation

The blocked C3-Mab microtiter plates (prepared in Example 2) were treated with 250 μl/well of either DFP (diisopropylfluorophosphate; 10 μg/ml in 0.14 M NaCl; Sigma Chem. Co., St. Louis, MO), or pAPMSF [(p-amidinophenyl)methanesulfonyl fluoride; 10 μg/ml, in 0.1 M sodium acetate, pH 6.0; Chemicon, El Segundo CA] for 30 minutes at 4°C. The wells then were washed with dilution buffer (0.05 M Hepes, pH 7.24, 0.2 M NaCl, 0.05 M benzamidine, 0.02 M EDTA, 0.4% casein, 0.6% ovalbumin, 2% BSA, 0.04 % Na-azide, and 0.05% Tween-80; filtered with 0.2 μm pore size filter) to which soybean trypsin inhibitor had been added prior to benzamidine addition. To each well was added 300 μl washing buffer, and the plates were stored at 4°C

for no longer than one month.

ii. Experimental Samples

For assays of APC activity in plasma from baboons, blood samples of 1.8 ml were drawn directly into a 0.2 ml anticoagulant mixture of 0.3M benzamidine, 0.13M trisodium citrate, 0.1M Hepes, pH 6.8, and 0.02% sodium azide. The blood then was prepared by centrifugation (3000xg, 3 min, 4 C; within 60 minutes of blood drawing), frozen, and stored at -70°.

d. Assay Method

Prior to assay, APC standards and baboon samples were further diluted in a dilution microplate using one part sample and either 20 or 30 parts dilution buffer to reach a total volume of 210 or 310 $\mu$l respectively. Each plate had at least one series of purified APC standards in addition to the samples.

To capture APC and PC antigen, aliquots (50-200 $\mu$l) from the diluted APC standards and experimental samples were transferred to C3-Mab coated microtiter plates containing an appropriate volume of dilution buffer to yield a final quantity of fluid equal to the volume of buffer used to coat the plate with antibody. The plates were incubated at room temperature for 1-1.5 hours or overnight at 4°C with equivalent results. Following the adsorption step, washing buffer (250 ul) was added to remove the unbound proteins and benzamidine. The covered plates were vigorously shaken on a rocking table (2-5 minutes, 200-260 rpm) and then the washing buffer was removed by rapping the inverted plates on layers of clean paper towels for thorough removal of residual liquid. The washing cycle was repeated for at least five times within 30 min for most complete removal of the benzamidine and especially for removal of

contaminating plasma enzymes that could nonspecifically bind to the plate.

Following the last washing cycle, a synthetic oligopeptide chromogenic substrate for APC, S-2366 (0.45-1.0 mM in TBS, pH 8.0, 0.05% Na-azide) was added to the wells. S-2366 is <Glu-Pro-Arg-pNA (Kabi Diagnostica, Uppsala, Sweden), it was prepared aseptically at 4°C and filtered through a 0.2 μm filter. The lyophilized substrate was diluted to 4 mM using sterile water. Prior to the assay the substrate was further diluted to 0.4 mM using filtered washing buffer in a disposable sterile reagent tray. Hydrolysis of the substrate was monitored at 405 nm (or at 405/630 nm) over time and recorded using Bio-Tek Microplate Autoreaders EL 309 or EL 312 (Bio-Tek Instruments, Inc., Highland Park, Winooski, VT). In some experiments, substrates other than S-2366 for APC were used with satisfactory results, e.g., S-2238 (Kabi Vitrum, Uppsala, Sweden), and Spectrozyme PCa (American Diagnostica, Greenwich, CT). When the standard APC concentration range covered more than 3 orders of magnitude, the plates were read repeatedly over various appropriate periods of time. In between readings, the plates were sealed and stored in wet chambers to avoid evaporation of liquid from the wells. Normal plasma levels of APC were measured by developing the plates at 4°C in most experiments.

Special care was taken to avoid microbial and enzyme contamination of the samples, buffers, microplates and substrate. Sterile water, plasticware, gloves and mask were used since microbial and other contaminating enzymes hydrolyze chromogenic substrates resulting in background activity. The enzyme assay step could be repeated by removing the hydrolyzed substrate and introducing fresh S-2366 solution into

the wells. The bound APC retained its amidolytic activity for weeks at 4°C. Sample results of this assay are set forth in Figure 2.

3. Chromogenic Assay

To determine whether pharmacologic elevation of circulating APC depletes circulating PC pool, PC zymogen activity was determined using a chromogenic assay for PC [Geiger, et al., Thromb. Haemost. 61: 86-92 (1989)]. (Data not shown.)

4. Platelet Assay

Autologous baboon blood platelets were labeled with $^{111}$In-oxine according to the following procedures. Whole blood (100 ml) was collected directly into plastic bags (TA-3, Fenwal Labs, Deerfield, IL) containing 20 ml acid-citrate-dextrose anticoagulant (NIH Formula A). The blood was centrifuged in the bag at 300 x g for 10 minutes. The supernatant platelet-rich plasma (PRP) was then transferred to a second bag and the pH adjusted to 6.5 by the addition of 0.15M citric acid (0.1ml/10ml PRP). The red blood cell fraction was returned to the donor mammal. The platelets were formed into a pellet by centrifugation of the PRP at 1300 x g for 15 minutes. The supernatant platelet-poor plasma (PPP) was completely decanted and discarded.

To remove residual plasma proteins, the bag containing the platelet pellet was carefully washed once by overlaying with 30 ml of Ringer's citrate dextrose (RCD, pH 6.5) that was then decanted and discarded. The pellet was then gently resuspended in 5.0 ml RCD, and incubated for 30 minutes with 500-700 micro Ci $^{111}$In-oxine (Amersham Corp., Arlington Heights, IL). Contaminating red cells were removed by a final slow centrifugation at 200 x g for 5 minutes.

Labeling efficiency was determined by diluting

200 microliters of the labeled-platelet concentrate with 5.0 ml RCD, and comparing the activity in 0.5 ml of the diluted platelet suspension with the activity in 0.5 ml of cell-free supernatant following centrifugation at 3000 x g for 30 minutes. A measured volume of labeled platelet suspension containing approximately 13 percent non-platelet bound isotope was then injected directly into the recipient mammals following the preparation of a 100 microliter standard. Additional washing procedures to remove non-platelet bound isotope were deemed undesirable since they may produce *in vitro* cell damage.

Circulating platelet $^{111}$In-activity was determined from 4 ml blood samples drawn prior to and following graft placement, and collected in 2 mg/ml (ethylenedinitrilo)-tetraacetic acid (EDTA). One ml of each sample was used for platelet counting, and 1.0 ml was counted for whole blood $^{111}$In-activity. The remaining 2 ml were centrifuged at 3000 x g for 30 minutes, and 1.0 ml of the supernatant (PPP) was counted for plasma $^{111}$In-activity. All blood and plasma samples were counted using a gamma spectrometer (Nuclear Chicago, Chicago, IL). Platelet counts were performed on whole blood using a electronic platelet counter (Clay Adams UF-100, Parsippany, NJ).

Scintillation camera imaging of both gamma photon peaks of $^{111}$In (172 keV and 247 keV) has generally required high energy collimation to prevent image blurring, despite a decreased in both sensitivity and spatial resolution. Since platelet-specific activity was not a limiting factor in the present studies, a high sensitivity $^{99}$Tc collimator could be used with good resolution by imaging only the lower energy peak of $^{111}$In (172 keV peak and with a 15 percent energy window). Images of the Dacron grafts, including

proximal distal Silastic segments, were acquired with a Picker DC 4/11 Dyna scintillation camera (Picker Corp., Northford, CT) and stored on and analyzed by a Medical Data System SIMUL computer (Medtronic, Ann Arbor, MI) interfaced the camera. This system permitted simultaneous acquisition and analysis of data in 64 x 64 word mode and was used to generate the data shown in Figure 3. Immediately prior to imaging the graft segments *ex vivo*, 2 minute images were acquired of the 200 microliter sample of platelet concentrate (injection standard) and of 4.0 mm i.d.. Silastic tubing filled with autologous blood and having the same luminal volume as the graft segment (blood standard). Results of the platelet assays are diagrammed in Figures 3 and 4.

5. Fibrin Assay

The baboons were injected with $^{125}$I-labeled fibrinogen (FGN) (0.005mCi) before each experiment. The grafts were removed if occlusion occurred before 60 minutes from the beginning of the experiment and were washed and stored in 2.5% glutaraldehyde. $^{125}$I-labeled FGN radioactivity was measured in counts per minute (cpm) after allowing the graft $^{111}$In activity (from the labeled autologous platelets) to decay for at least 30 days. At 30 days, the remaining platelet-bound $^{111}$In radioactivity was 0.0006% of the value at 0 minutes, compared with 70% of $^{125}$I radioactivity that remained at 30 days. The remaining $^{111}$In radioactivity was subtracted from the total radioactivity and total fibrin deposition was calculated by dividing this value by the plasma radioactivity determined at the same time as the graft radioactivity (cpm/ml) and multiplying by the circulating FGN concentration (mg/ml). (See Gruber, et al., Circulation 82(2): 578-85 (1990).) Results of the fibrin assay are set forth

in Figure 5.

6. Activated Partial Thromboplastin Time (APTT) Coagulation Assay

The assays were performed essentially as described in Gruber et al., Blood 73: 639-642 (1989). Briefly, 100 $\mu$l of fresh baboon plasma prepared within 10 minutes of drawing the blood sample was mixed with 100$\mu$l of APTT reagent (Thrombosil, Ortho Diagnostics, Raritan, NJ) and incubated for three minutes at 37°C. Clotting time was measured after recalcification using 100$\mu$l of 25mM $CaCl_2$ in a fibrometer (BBL Fibrosystem).

7. Thrombosis study

As illustrated in Tables 3 and 4 below, when there was no thrombogenic graft placed into the shunt, the two doses of thrombin infusions (1 and 2 U/kg/min) induced the generation of 254 ng/ml (4nM) of circulating APC and 409 ng/ml (6.6 nM) of circulating APC by 60 minutes, respectively (mean values). (See Tables 3 and 4 below.) When thrombin was infused at a dose of 2 U/kg/hr and a thrombogenic device was placed into the shunt, the levels of induced endogenous APC activity reached 760 ng/ml (12.3 nM), as illustrated in Table 6.

Similar, pharmacologically efficient levels of APC activity were achieved formerly by infusion of exogenous purified APC into baboons with the purpose of antithrombotic therapy. (See Gruber, et al., Blood 73: 639-642 (1989); Gruber, et al., Circulation 82: 578-585 (1990).) Our endogenous APC experiments demonstrated unexpected increases in APC levels, however. The increase in APC levels following infusion at the higher dose of 2 U/kg/min of thrombin was accompanied by the prolongation of activated partial thromboplastin time by 70 seconds after 30 minutes and 157 seconds after 60 minutes (see Table

6). The activated partial thromboplastin time (APTT) returned to quasi-normal (41.9 seconds) 3 hours after termination of thrombin infusion (not shown). In Figure 6, data from animals with thrombogenic devices present in the shunt is illustrated and demonstrates the prolongation in APTT produced by higher doses of thrombin.

The inhibition of thrombus formation was comparable to the inhibition of thrombosis during infusion of exogenous purified human plasma derived APC into baboons at similar plasma APC levels. (See Gruber, et al., Blood 73: 639-642 (1989) and Gruber, et al., *supra* (1991). Control thrombogenic grafts accumulated 4.65 ± 2.01 billion platelets within one hour, the thrombogenic chambers accumulated 2.15 ± 1.16 billion platelets. (See Table 2.) IV infusion of 1 U kg/min of α-thrombin inhibited platelet deposition in the graft by 49% (2.38 ± 1.0 billion platelets deposited) and by 97% in the chamber (0.15 billion platelets deposited). (See Table 5.) The 2 U/kg/min thrombin infusion inhibited platelet deposition by 62% (1.78 ± 0.69 billion platelets deposited) and 99% (0.03 billion platelets deposited) in the graft and the chamber, respectively. See also Figure 8, in which the percent increase in circulating APC and its effect on platelet deposition is illustrated.

Circulating PC was activated by IV infusion of α-thrombin and levels of APC averaged 2.6 ng/ml and 3.3 ng/ml before thrombin infusion and 276 ng/ml (N=4) and 498 ng/ml (N=5) at 60 minutes of thrombin infusion, respectively. (See Figure 2.)

Infusion of thrombin at the two described doses did not induce detectable thrombosis. Thrombus formation in the thrombogenic devices resulted in about an 8% drop in platelet count in controls (see

Table 2). In the 1 U kg/min thrombin infusion experiments circulating platelet values were 336,000/μl before infusion and 318,000/μl after infusion (5.4% platelet drop), thus this dose of thrombin did not cause, but rather prevented, platelet consumption (see Table 5). Arterial-type thrombus formation was distinctly inhibited, and venous-type thrombus formation was abolished. Figures 3 and 4 illustrate the significant decrease in platelet deposition in both segments of the graft. Figure 5 illustrates the decrease in fibrin deposition. Infusion of 2 U/kg/min thrombin for one hour reduced platelet counts by 30% versus controls without significant increase in antithrombotic effect (see Table 6). This suggests that 1U/kg/min thrombin induced near maximal antithrombotic effect. Protein C zymogen level was not depleted during one hour infusion of 1 U/kg/min thrombin (-3%, from 77.3 % to 75%). Bleeding times were not prolonged significantly at the 2 U/kg/min thrombin dose (from 3.1 ± 0.6 minutes to 4 ± 0.7 minutes). No adverse effects were observed during and after one hour thrombin infusion at either dose.

TABLE 2

**Control study: thrombogenic device present; no thrombin added**

| Animal: | 1507 | 1637 | 1646 | 1641 | 1448 | 9006 | MEAN±SD |
|---|---|---|---|---|---|---|---|
| Date: | 3/7/91 | 5/30/91 | 6/5/91 | 6/7/91 | 4/9/91 | 2/22/91 | |
| **Platelet count (X $10^3/\mu l$)** | | | | | | | |
| 0 min. | 416 | 375 | 327 | 311 | 355 | 490 | 379±65 |
| 60 min. | 448 | 324 | 337 | 288 | 297 | 411 | 350±64 |
| **Activated partial thromboplastin time (APTT, SEC)** | | | | | | | |
| 0 min. | 37.1 | 29.4 | 30.9 | 28.4 | ND* | ND | 31.5±3.9 |
| 60 min. | 37.9 | 29.9 | 31.9 | 28.9 | ND | ND | 32.2±4.0 |
| **Activated Protein C (APC, ng/ml)** | | | | | | | |
| 0 min. | 13.8 | 14 | 8 | 1.9 | ND | 3.3 | 8.2±5.6 |
| 30 min. | 11.4 | ND | ND | ND | ND | ND | ND |
| 60 min. | 43.8 | 14.1 | 18.2 | 13.2 | ND | 17.5 | 21.4±12.7 |
| **Platelets deposited (X $10^9$)** | | | | | | | |
| **Dacron** | | | | | | | |
| 10 min. | 0.29 | 0.58 | 0.48 | 0.21 | 0.18 | 0.64 | 0.4 |
| 20 min. | 1.08 | 1.44 | 1.64 | 1.02 | 0.63 | 2.24 | 1.34 |
| 30 min. | 1.85 | 2.58 | 3.08 | 1.87 | 1.19 | 4.01 | 2.43 |
| 40 min. | 2.98 | 3.45 | 4.32 | 2.67 | 1.88 | 5.81 | 3.52 |
| 50 min. | 3.34 | 4.02 | 5.45 | 3.22 | 2.42 | 6.94 | 4.23 |
| 60 min. | 3.04 | 4.32 | 6.5 | 3.59 | 2.73 | 7.69 | 4.65 |
| **Chamber** | | | | | | | |
| 10 min. | 0.06 | 0.02 | 0 | 0.02 | 0 | 0 | 0.02 |
| 20 min. | 0.12 | 0.03 | 0.05 | 0.04 | 0.03 | 0.04 | 0.05 |
| 30 min. | 0.19 | 0.24 | 0.34 | 0.14 | 0.14 | 0.29 | 0.22 |
| 40 min. | 0.47 | 0.82 | 1.01 | 0.08 | 0.4 | 1.17 | 0.66 |
| 50 min. | 1.39 | 1.33 | 1.85 | 0.2 | 1.13 | 2.75 | 1.44 |
| 60 min. | 2.12 | 2.26 | 2.37 | 0.48 | 1.61 | 4.05 | 2.15 |

| Fibrin deposited at 60 min. (mg) | | | | | | | |
|---|---|---|---|---|---|---|---|
| Dacron | 1.69 | 2.17 | 2.7 | 2.95 | 2.04 | 3.16 | 2.45±0.57 |
| Chamber | 3.73 | 5.03 | 2.41 | 2.84 | 2.74 | 3.59 | 3.39±0.95 |

* ND= not determined

********

TABLE 3

Thrombogenic device absent; 1U/kg/min. thrombin added

| Animal: | 9003 | 1607 | 1641 | MEAN±SD |
|---|---|---|---|---|
| Date: | 2/14/91 | 6/4/91 | 6/5/91 | |
| **Platelet count (X $10^3/\mu l$)** | | | | |
| 0 min. | 422 | 260 | 350 | 344±81 |
| 60 min. | 404 | 354 | 354 | 371±29 |
| **Activated partial thromboplastin time (APTT, SEC)** | | | | |
| 0 min. | 40.4 | 30.9 | 29.9 | 33.7±5.8 |
| 30 min. | 44.3 | 38.9 | 48.4 | 43.9±4.8 |
| 60 min. | 51.9 | 40.9 | 70.9 | 54.6±15.2 |
| 90 min. | 40.9 | 38.9 | 48.9 | 42.9±5.3 |
| **Activated protein C (APC, ng/ml)** | | | | |
| 0 min. | 4.9 | 6.4 | 6.3 | 5.8±0.8 |
| 30 min. | 133 | 298 | 287 | 239±92 |
| 60 min. | 146 | 309 | 308 | 254±94 |
| 90 min. | 36 | 72 | 95 | 68±30 |
| 240 min. | ND* | 5 | 8 | 6.5±2.1 |

* ND = not determined

TABLE 4

**Thrombogenic device absent; 2U/kg/min. thrombin added**

| Animal: | 1508 | 9006 | 1256 | MEAN±SD |
|---|---|---|---|---|
| Date: | 3/5/91 | 4/19/91 | 5/2/91 | |
| **Platelet count (X $10^3/\mu l$)** | | | | |
| 0 min. | 670 | 520 | 152 | 447±267 |
| 60 min. | 585 | 401 | 127 | 371±230 |
| **Activated partial thromboplastin time (APTT, SEC)** | | | | |
| 0 min. | 32.2 | 32.9 | 31.4 | 32.2±0.75 |
| 30 min. | 57.9 | ND* | 54.9 | 56.4±2.1 |
| 60 min. | 100.9 | ND | 147 | 124±32.6 |
| 90 min. | ND | ND | 64.4 | 64.4 |
| **Activated protein C (APC, ng/ml)** | | | | |
| 0 min. | 1 | 2.4 | 8.8 | 4.1±4.2 |
| 30 min. | 312 | 430 | 351 | 364±60 |
| 60 min. | 310 | 500 | 417 | 409±95 |
| 90 min. | 72 | 120 | 144 | 112±37 |
| 240 min. | ND | ND | 7.8 | 7.8 |

* ND = not determined

TABLE 5

**Thrombogenic device present; 1U/kg/min. thrombin added**

| Animal: | 1637 | 1641 | 1646 | 1610 | MEAN±SD |
|---|---|---|---|---|---|
| Date: | 6/5/91 | 6/6/91 | 6/6/91 | 6/25/91 | |
| **Platelet count (X $10^3/\mu l$)** | | | | | |
| 0 min. | 331 | 245 | 295 | 474 | 336±98 |
| 60 min. | 292 | 303 | 305 | 373 | 318±37 |
| **Activated partial thromboplastin time (APTT, SEC)** | | | | | |
| 0 min. | 29.4 | 31.4 | 33.9 | 30.9 | 31.4±1.9 |
| 30 min. | 41.4 | 40.9 | 58.4 | 41.4 | 45.5±8.6 |
| 60 min. | 55.4 | 57.4 | 79.9 | 48.9 | 60.4±13.5 |
| 90 min. | 41.9 | 44.4 | 54.9 | 43.9 | 46.3±5.9 |
| **Activated protein C (APC, ng/ml)** | | | | | |
| 0 min. | 1.4 | 4.4 | 2.5 | 2 | 2.6±1.3 |
| 30 min. | 180 | 215 | 352 | 160 | 227±87 |
| 60 min. | 221 | 267 | 359 | 256 | 276±59 |
| 90 min. | 50 | 69 | 114 | 100 | 83±29 |
| 240 min. | 2.7 | 7.1 | 6.9 | 4.2 | 5.2±2.1 |
| **Fibrin deposited at 60 min. (mg)** | | | | | |
| Dacron | 1.65 | 1.11 | 1.27 | 2.57 | 1.65±0.65 |
| Chamber | 0.43 | 0.25 | 0.34 | 1.01 | 0.51±0.34 |

| (Animal: | 1637 | 1641 | 1646 | 1610 | MEAN±SD) |
|---|---|---|---|---|---|
| **Platelets deposited (X $10^9$)** | | | | | |
| **Dacron** | | | | | |
| 10 min. | 0.38 | 0.33 | 0.68 | 0.52 | 0.48±0.16 |
| 20 min. | 1.12 | 1.14 | 1.91 | 1.72 | 1.47±0.4 |
| 30 min. | 1.73 | 1.52 | 2.46 | 2.89 | 2.15±0.64 |
| 40 min. | 1.89 | 1.72 | 2.49 | 3.56 | 2.42±0.83 |
| 50 min. | 1.92 | 1.78 | 2.37 | 3.65 | 2.43±0.85 |
| 60 min. | 1.87 | 1.62 | 2.2 | 3.83 | 2.38±1 |
| **Chamber** | | | | | |
| 10 min. | 0.03 | 0 | 0.04 | 0.09 | 0.04±0.04 |
| 20 min. | 0.01 | 0.05 | 0.07 | 0.16 | 0.07±0.06 |
| 30 min. | 0.05 | 0.07 | 0.13 | 0.26 | 0.13±0.09 |
| 40 min. | 0 | 0.03 | 0.14 | 0.43 | 0.15±0.2 |
| 50 min. | 0 | 0.06 | 0.09 | 0.52 | 0.17±0.24 |
| 60 min. | 0 | 0.08 | 0.05 | 0.46 | 0.15±0.21 |

TABLE 6

**Thrombogenic device present; 2U/kg/min. thrombin added**

| Animal: | 1507 | 9006 | 1637 | 1609 | 1506 | MEAN±SD |
|---|---|---|---|---|---|---|
| Date: | 3/8/91 | 4/24/91 | 5/31/91 | 6/21/91 | 6/18/91 | |
| **Platelet count (X $10^3/\mu l$)** | | | | | | |
| 0 min. | 488 | 449 | 358 | 313 | 338 | 389±75 |
| 60 min. | 461 | 273 | 236 | 193 | 195 | 272±111 |
| **Activated partial thromboplastin time (APTT, SEC)** | | | | | | |
| 0 min. | 34.9 | 32.9 | 29.4 | 31.9 | 32.2 | 32.3±2 |
| 30 min. | ND* | 88.9 | 45.4 | 90.9 | 53.9 | 70±24 |
| 60 min. | 198.2 | 262 | 73.9 | 139 | 110.5 | 157±74 |
| 90 min. | ND | 92.9 | 50.9 | 129 | 59.9 | 83±35 |
| **Activated protein C (APC, ng/ml)** | | | | | | |
| 0 min. | 5 | 2.3 | 3.6 | 3.1 | 2.5 | 3.3±1.1 |
| 30 min. | 590 | 302 | 374 | 420 | 135 | 364±166 |
| 60 min. | 725 | 350 | 375 | 760 | 282 | 498±226 |
| 90 min. | 196 | 74 | 108 | 275 | 40 | 139±96 |
| 240 min. | ND | 18 | 8.5 | 6 | 9.1 | 10.4±5.2 |
| **Fibrin deposited at 60 min. (mg)** | | | | | | |
| Dacron | 1.02 | 0.71 | 1.96 | 1.15 | 1.23 | 1.22±0.46 |
| Chamber | 0.24 | 0.07 | 0.31 | 0.52 | 0.82 | 0.39±0.29 |

| (Animal: | 1507 | 9006 | 1637 | 1609 | 1506 | MEAN±SD) |
|---|---|---|---|---|---|---|
| **Platelets deposited (X $10^9$)** | | | | | | |
| **Dacron** | | | | | | |
| 10 min. | 0.42 | 0.98 | 0.31 | 0.49 | 0.31 | 0.5±0.28 |
| 20 min. | 1.44 | 2.46 | 1.02 | 1.43 | 0.73 | 1.42±0.66 |
| 30 min. | 2.19 | 3.72 | 1.51 | 1.95 | 1.14 | 2.1±0.99 |
| 40 min. | 1.84 | 3.84 | 1.99 | 1.81 | 1.17 | 2.13±1.01 |
| 50 min. | 1.73 | 3.24 | 2.07 | 1.56 | 1.15 | 1.95±0.79 |
| 60 min. | 1.58 | 2.84 | 2.05 | 1.26 | 1.15 | 1.78±0.69 |
| **Chamber** | | | | | | |
| 10 min. | 0.04 | 0.00 | 0.00 | 0.00 | 0.03 | 0.014±0.02 |
| 20 min. | 0.16 | 0.00 | 0.00 | 0.06 | 0.02 | 0.048±0.07 |
| 30 min. | 0.19 | 0.00 | 0.00 | 0.05 | 0.09 | 0.066±0.08 |
| 40 min. | 0.17 | 0.00 | 0.00 | 0.00 | 0.04 | 0.042±0.07 |
| 50 min. | 0.15 | 0.00 | 0.00 | 0.00 | 0.00 | 0.030±0.07 |
| 60 min. | 0.14 | 0.00 | 0.00 | 0.00 | 0.00 | 0.028±0.06 |

* ND = not determined

Because thrombin itself clots blood and is capable of inducing thrombosis (it is "prothrombotic") via activating platelets and factors V and VIII, and via clotting fibrinogen, reduction or abolition of thrombus formation on thrombogenic surfaces during thrombin infusion was a novel finding that contradicted previous, "common" knowledge about thrombin. (See, e.g., Walz, et al., eds., "Bioregulatory functions of thrombin", in Ann. N.Y. Acad. Sci. 485: 5-413 (1986).) In fact, many current antithrombotic therapies are based on inhibiting thrombin. The results presented here indicate that endogenous APC generated upon infusion of low levels of thrombin actually prevents thrombosis that was expected to arise due to thrombin infusion and also prevents thrombus formation on thrombogenic surfaces. A detectable increase in platelet consumption at the higher thrombin dose suggested that the APC generated during thrombin infusion could not protect from the prothrombotic effect of thrombin infusion exceeding certain dose limits (i.e., 2 U/kg/min). The ability of any agent to induce increases in the concentration of the antithrombotic circulating endogenous APC and its potential to be antithrombotic can be determined using the enzyme capture assay (ECA) that specifically measures the active APC from blood.

8. Protein C Zymogen

Another useful method of increasing circulating APC levels comprises the infusion of protein C zymogen. It is reported herein that protein C zymogen levels correlate with APC levels; see, e.g., Fig. 9. Furthermore, infusion of protein C into homozygous protein C deficient patients A and B with severe thrombophilia increases circulating protein C levels (see Tables 7 and 8 below).

TABLE 7

Coagulation Parameters in Patient A with Homozygous Protein C Deficiency Following Protein C (PC) Concentrate Administration at a Dose of 80 Units/kg Body Weight

| | PC Ag (% of normal) | PC Act (% of normal) | PCP (pM) | APC (pM) | F1+2 (nM) | FPA (nM) |
|---|---|---|---|---|---|---|
| Pre-Infusion | 16 | 18 | 0 | 5.8 | 2.15 | 4.82 |
| Post-Infusion | | | | | | |
| 30 min. | 295 | 267 | 2.89 | 65.0 | 1.92 | 2.12 |
| 1 hr. | 235 | 248 | - | 61.7 | 1.84 | 1.82 |
| 2 hr. | 210 | 227 | 2.57 | 53.3 | 1.90 | 1.47 |
| 4.5 hr. | 185 | 201 | - | 40.0 | 1.95 | 4.11 |
| 6.5 hr. | 162 | 179 | 2.14 | 41.7 | 1.81 | 1.15 |
| 11 hr. | 142 | 123 | - | 29.2 | 1.34 | 1.47 |
| 24 hr. | 95 | 88 | - | 15.0 | 1.12 | 2.45 |
| 30 hr. | 69 | 63 | 0.93 | 13.3 | 1.20 | 1.73 |
| 44 hr. | 42 | 43 | 1.00 | - | 1.01 | 4.84 |
| 68 hr. | 27 | 28 | - | - | 1.50 | - |

TABLE 8

Coagulation Parameters in Patient B with Homozygous Protein C Deficiency Following Protein C (PC) Concentrate Administration at a Dose of 80 Units/kg Body Weight

| | PC Ag (% of normal) | PC Act (% of normal) | PCP (pM) | APC (pM) | F1+2 (nM) | FPA (nM) |
|---|---|---|---|---|---|---|
| Pre-Infusion | 23 | 21 | 2.21 | 3.2 | 4.26 | 5.93 |
| Post-Infusion | | | | | | |
| 30 min. | 285 | 220 | 4.26 | 46.7 | 3.96 | 1.15 |
| 1 hr. | 238 | 200 | - | 40.0 | 3.18 | 1.34 |
| 2 hr. | 224 | 186 | 3.74 | 39.3 | 2.46 | 1.99 |
| 4 hr. | 201 | 154 | - | 33.6 | 1.80 | 0.77 |
| 6 hr. | 166 | 135 | 1.89 | - | 1.50 | 0.63 |
| 17 hr. | 123 | 99 | 1.72 | 22.0 | 1.08 | 1.63 |
| 25 hr. | 100 | 77 | 1.20 | 16.8 | 1.32 | 3.44 |
| 42 hr. | 71 | 48 | 1.63 | - | 1.62 | 1.82 |
| 66 hr. | 60 | 34 | 1.00 | 9.7 | 1.47 | 1.24 |
| 90 hr. | 49 | 29 | 1.50 | 11.5 | 1.92 | 2.35 |

In addition, we have now performed a first preclinical trial with site-directed mutant thrombin K52E (chymotrypsin numbering: K60fE). As of June 1992, 2400 amidolytic units (units of Fenton's 98% amidolytically active α-thrombin) of K52E was infused intravenously over one hour (0 minutes to 60 minutes) into a 10kg young male baboon bearing thrombogenic grafts in a chronic AV shunt. In controls, the proximal graft generally accumulates arterial type thrombi while the distal graft accumulates venous type thrombi. About 10% of control grafts occlude within about 60 minutes and cannot be evaluated.

We inserted the grafts into the shunt and began the infusion of K52E distal to the graft; the distal end of the shunt was placed into the inferior vena cava. It was estimated that the K52E in the venous blood had about 1-2 seconds to reach the pulmonary capillary bed. During infusion of K52E the blood flow started to gradually increase and stabilized at 120 ml/min by 15-20 minutes into the experiment. At 60 minutes infusion of thrombin was completed, the graft was removed, and the flow in the shunt was restored. Bleeding time was about 5.5 min at 60 minutes, with no evidence of discomfort (e.g., hyperventilation or excessive mobility) or toxicity apparent in the animal. The shunt was subsequently removed surgically. The initial results observed are set forth below in Table 9.

Table 9

| Parameter tested | Time (minutes) 0′ | 30′ | 60′ | 75′ | 90′ | 120′ |
|---|---|---|---|---|---|---|
| APTT (sec) | 31.5 | 71.4 | 135 | 100 | 66.4 | 50.9 |
| APC (ng/ml) | 10.4 | 484 | 417 | 181 | 124 | 41.3 |
| PC activity (%) | 54 | 51 | 40 | 40.7 | 40.7 | 40.4 |
| F1-2 (nmol/l) | 0.45 | | 1.5 | | | |
| TAT (ng/ml) | 24.2 | | 125.8 | | | |
| FPA | 7 | | 74 | | | |
| FGN (mg/ml) | 3.1 | | 2.68 | | | |
| Platelet count/nl | 250 | | 210 | | | |
| Platelets in Dacron $x10^{-9}$ | 0.078(5′) | | 0.909 | | | |
| Platelets in Teflon $x10^{-9}$ | 0.385(5′) | | 0.400* | | | |
| Fibrin in Dacron (mg) | ND | | 1.18 | | | |
| Fibrin in Teflon (mg) | ND | | 1.63 | | | |

* The infusion was delayed for 5 minutes because of technical difficulties. Ninety-seven percent (97%) of the platelets in the Teflon chamber deposited during this delay.

The baboon platelet aggregating activity of K52E was about 20% of α-thrombin. For comparison purposes, the following data were gathered previously in six (6) similar size baboons receiving 1200 amidolytic units of α-thrombin under similar experimental conditions (mean values), as illustrated in Table 10 below.

Table 10

| Parameter tested | Time (minutes) 0′ | 30′ | 60′ | 75′ | 90′ | 120′ |
|---|---|---|---|---|---|---|
| APTT (sec) | 31 | | 148 | | | |
| APC (ng/ml) | 4 | | 415 | | | |
| PC activity (%) | 97.6 | | 80.8 | | | |
| F1-2 (nmol/l) | | | ND | | | |
| TAT (ng/ml) | 24 | | 131 | | | |
| FPA | 4.5 | | 204 | | | |
| FGN (mg/ml) | 3.82 | | 2.46 | | | |
| Platelet count/nl | 389 | | 271 | | | |
| Platelets in Dacron $x10^{-9}$ | 0.50(10′) | | 1.77 | | | |
| Platelets in Teflon $x10^{-9}$ | 0.01(10′) | | 0.03 | | | |
| Fibrin in Dacron (mg) | ND | | 1.23 | | | |
| Fibrin in Teflon (mg) | ND | | 0.82 | | | |

Thus, K52E at a 2400 U dose resulted in less circulating FPA by 60 minutes than α-thrombin at a 1200 U dose. Despite the fact that the baboon receiving K52E proved to be partially deficient in PC zymogen (50% levels), it produced over 400 ng/ml of circulating APC. The APTT values corresponded to this APC level. This baboon might have produced APC levels reaching 900 ng/ml if the PC zymogen was normal. Fibrinogen and platelet consumption was negligible with K52E. The 3-fold increase in prothrombin fragment F1-2 suggested efficacious inhibition of prothrombin cleavage. K52E at this dose very efficaciously inhibited both arterial type (Dacron platelets, 78% inhibition) and venous type (Teflon

platelets, 96% inhibition) thrombus formation without adverse effects (i.e., bleeding or toxicity at a very high dose).

Therefore, the above data indicate that increasing the circulating concentration of the zymogen protein C will increase the circulating levels of APC. The data also suggest that any agent that increases the activation of protein C, thus increasing the level of APC, could be used in conjunction with infusion of the purified zymogen for (a) preventing the depletion of the endogenous zymogen pool during the induced anticoagulant therapy, and (b) reaching higher levels of APC.

We conclude that pharmacologic activation of the endogenous protein C system results in a potent antithrombotic effect, and the approach of pharmacological elevation of circulating APC levels will be clinically useful. We also conclude that any agent, administered orally or parenterally at appropriate dosages, that increases the concentration of endogenous APC in circulating blood can be antithrombotic and useful in the treatment and/or prevention of intravascular thrombus formation.

9. Methods of Preparing Useful Compositions

The present invention also contemplates various methods of preparing compositions useful in the inhibition of thrombosis formation in an individual. In one such method, an agent capable of increasing the blood activated protein C level in an individual is admixed with a pharmaceutically acceptable carrier or excipient. In one variation, the agent is capable of increasing the blood activated protein C level via enzymatic cleavage of protein C. The agent may comprise a serine protease, such as thrombin, and more preferably, human thrombin. The thrombin used may be

selected from the group comprising plasma-derived or recombinant α-thrombin, thrombin E192Q, thrombin K52E, and active site acylated-thrombin, to name a few examples.

The effective amount of agent included in the composition may vary according to the therapeutic modality, the needs of the individual, and the recommendations of the physician. In one variation, e.g. when the therapeutic agent comprises thrombin, an effective amount of thrombin is in the range of from 0.05U/kg/min to 2U/kg/min. The compositions of the present invention may further be formulated so that the effective amount of the activated protein C level-increasing agent -- such as thrombin -- may be administered over a period of at least 20 minutes. In one embodiment, an effective amount of the therapeutic agent, such as thrombin, is sufficient to achieve an activated protein C concentration in the blood of an individual three to five times the normal level of activated protein C. In another variation, the effective amount of agent (e.g., thrombin) is sufficient to achieve an activated protein C concentration in the blood of the individual of from 10 to 760 ng/ml. It may also be preferred that the effective amount of agent (e.g., thrombin) is sufficient to maintain the activated protein C concentration for a time period of at least 30 minutes. Compositions according to the present invention may be formulated for intravenous administration, or for continuous or bolus injection.

Another variation includes the admixture of an antithrombotic agent into the composition. In various embodiments, the antithrombotic agent is selected from the group comprising fibrinolytic agents, oral anticoagulants, dextrans, pentoxifilline, snake venom,

soluble thrombomodulin, antiplatelet drugs, cyclooxygenase inhibitors, cAMP modulators, ticlopidine, thrombolytic agents, streptokinase, Eminase, urokinase, tissue plasminogen activator, anticoagulant peptides, protein C, and activated protein C.

The present invention also contemplates methods of preparing compositions formulated for administration to individuals suffering from surgically induced thrombosis, or for administration substantially concurrently with exposing the blood of an individual to a prosthetic surface on a prosthetic device. Examples of prosthetic devices include (a) a cardiopulmonary assist device; (b) a hemodialysis device; (c) an arterial prosthesis; (d) a venous prosthesis; (e) an arteriovenous shunt; (f) a cardiopulmonary bypass oxygenator and/or pump apparatus; (g) heart valve implants or artificial heart valves; and (h) an artificial heart.

The foregoing is intended to be illustrative of the present invention, but not limiting. Numerous variations and modifications may be effected without departing from the true spirit and scope of the invention.

We Claim:

1. A therapeutic composition suitable for administration via injection, comprising an effective amount of an agent capable of increasing the blood activated protein C level via enzymatic cleavage of protein C in a pharmaceutically acceptable carrier or excipient.

2. The composition of claim 1, wherein said effective amount of said agent is administered at the dosage of 0.05U/kg/min to 2U/kg/min.

3. The composition of claim 1, wherein said agent is present in a solution of up to 0.5U agent per ml of carrier.

4. The composition of claim 1, wherein said agent is infused over a period of at least 20 minutes.

5. The composition of claim 1, wherein said agent comprises thrombin.

6. A diagnostic composition comprising an effective amount of a thrombomodulin-dependent protein C activating agent in a pharmaceutically acceptable carrier or excipient.

7. The composition of claim 6, wherein said agent is administered at a suggested dosage of 0.1U/kg/min to 2U/kg/min.

8. The composition of claim 6, wherein said agent is present in a solution of up to 0.5U agent per ml of carrier.

9. The composition of claim 6, wherein said agent is infused over a period of at least 5 minutes.

10. The composition of claim 6, wherein said agent comprises thrombin.

11. A method of preparing a composition useful in the inhibition of thrombosis formation in an individual, comprising admixing an agent capable of increasing the blood activated protein C level in said

individual with a pharmaceutically acceptable carrier or excipient.

12. The method of claim 11, wherein said agent is capable of increasing the blood activated protein C level via enzymatic cleavage of protein C.

13. The method of claim 11, wherein said agent comprises a serine protease.

14. The method of claim 11, wherein said protease comprises thrombin.

15. The method of claim 11, wherein said thrombin comprises human thrombin.

16. The method of claim 14, wherein said thrombin is selected from the group comprising plasma-derived or recombinant α-thrombin, thrombin E192Q, thrombin K52E, and active site acylated-thrombin.

17. The method of claim 14, wherein said effective amount of thrombin is in the range of from 0.05U/kg/min to 2U/kg/min.

18. The method of claim 11, wherein said composition is formulated so that said effective amount of thrombin may be administered over a period of at least 20 minutes.

19. The method of claim 14, wherein said effective amount of thrombin is sufficient to achieve an activated protein C concentration in the blood of said individual three to five times the normal level of activated protein C.

20. The method of claim 14, wherein said effective amount of thrombin is sufficient to achieve an activated protein C concentration in the blood of said individual of from 10 to 760 ng/ml.

21. The method of claim 14, wherein said effective amount of thrombin is sufficient to maintain said activated protein C concentration for a time period of at least 30 minutes.

22. The method of claim 11, further comprising the admixture of an antithrombotic agent into said composition.

23. The method of claim 22, wherein said antithrombotic agent is selected from the group comprising fibrinolytic agents, oral anticoagulants, dextrans, pentoxifilline, snake venom, soluble thrombomodulin, antiplatelet drugs, cyclooxygenase inhibitors, cAMP modulators, ticlopidine, thrombolytic agents, streptokinase, Eminase, urokinase, tissue plasminogen activator, anticoagulant peptides, protein C, and activated protein C.

24. The method of claim 11, wherein said composition is formulated for intravenous administration.

25. The method of claim 24, wherein said composition is formulated for bolus injection.

26. The method of claim 11, wherein said composition is formulated for administration substantially concurrently with exposing the blood of said individual to a prosthetic surface on a prosthetic device.

27. The method of claim 26, wherein said prosthetic device is selected from the group consisting of:

(a) a cardiopulmonary assist device,

(b) a hemodialysis device,

(c) an arterial prosthesis,

(d) a venous prosthesis,

(e) an arteriovenous shunt,

(f) a cardiopulmonary bypass oxygenator and/or pump apparatus,

(g) heart valve implants or artificial heart valves, and

(h) an artificial heart.

28. The method of claim 11, wherein said thrombosis comprises surgically induced thrombosis.

29. A diagnostic method for determining the presence of functional thrombomodulin in an individual, comprising the steps of:

a) obtaining a first plasma sample from said individual;

b) determining the protein C level in a first portion of said sample;

c) determining the activated protein C level in a second portion of said sample;

d) intravenously injecting a thrombomodulin-dependent protein C activating agent into a vein of said individual for 5 minutes;

e) obtaining a second plasma sample from said individual;

f) determining the activated protein C level in said second sample; and

g) comparing the activated protein C levels determined for said first and second samples to determine the presence of functional thrombomodulin in said individual.

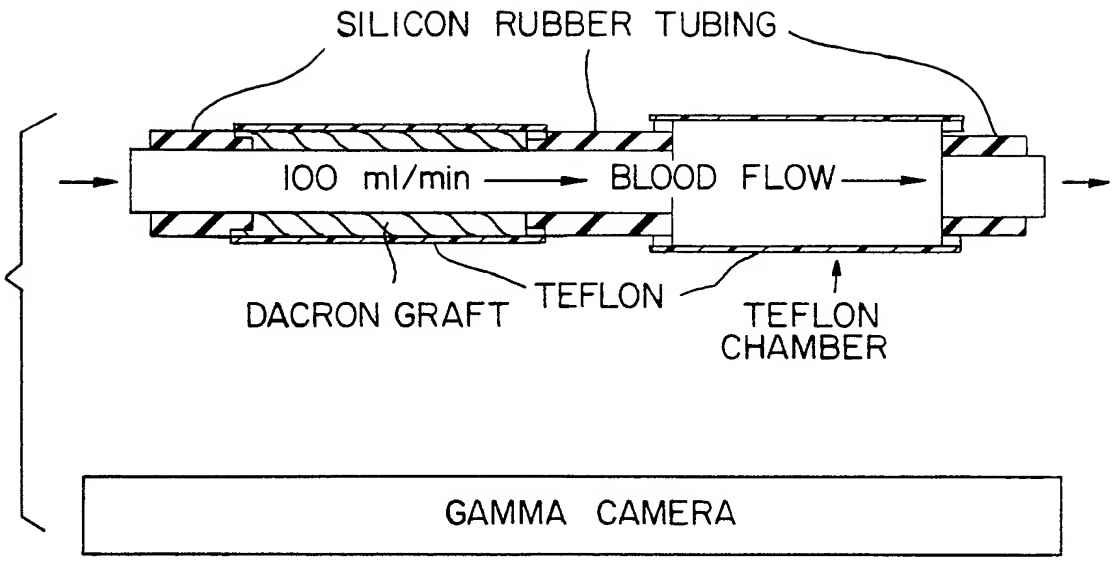
30. The method of claim 29, wherein said agent comprises a serine protease.

31. The method of claim 30, wherein said protease comprises thrombin.

32. The method of claim 29, further comprising administering said agent at a rate of 0.1U/kg/min.

33. A method of preparing a composition useful according to the method of claim 29, comprising admixing a protein C activating agent with a pharmaceutically acceptable carrier or excipient.

FIG. 1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DACRON GRAFT
TEFLON
TEFLON CHAMBER
GAMMA CAMERA

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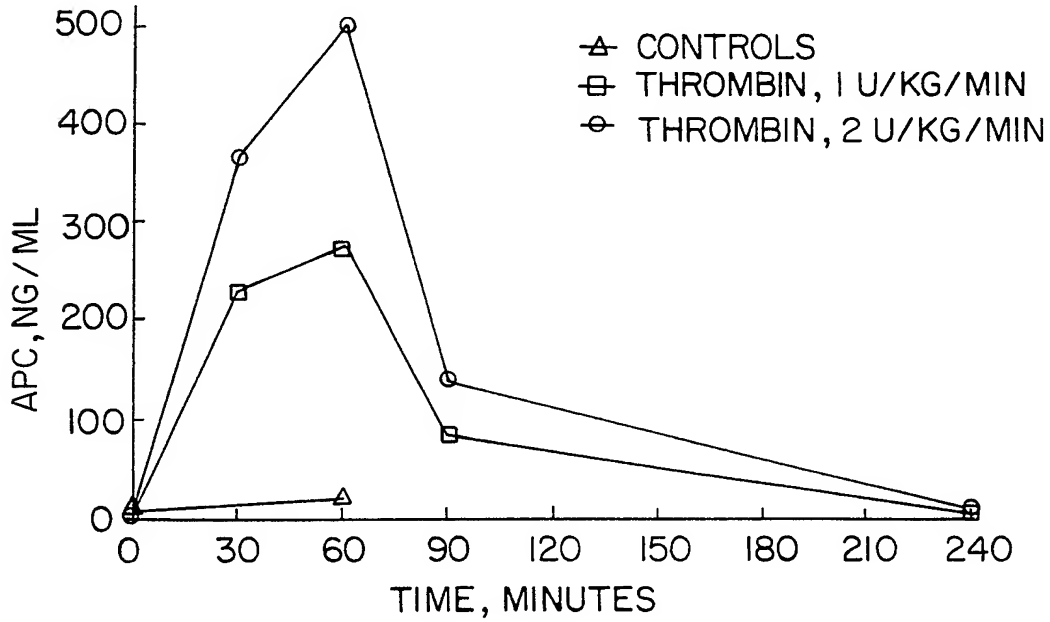
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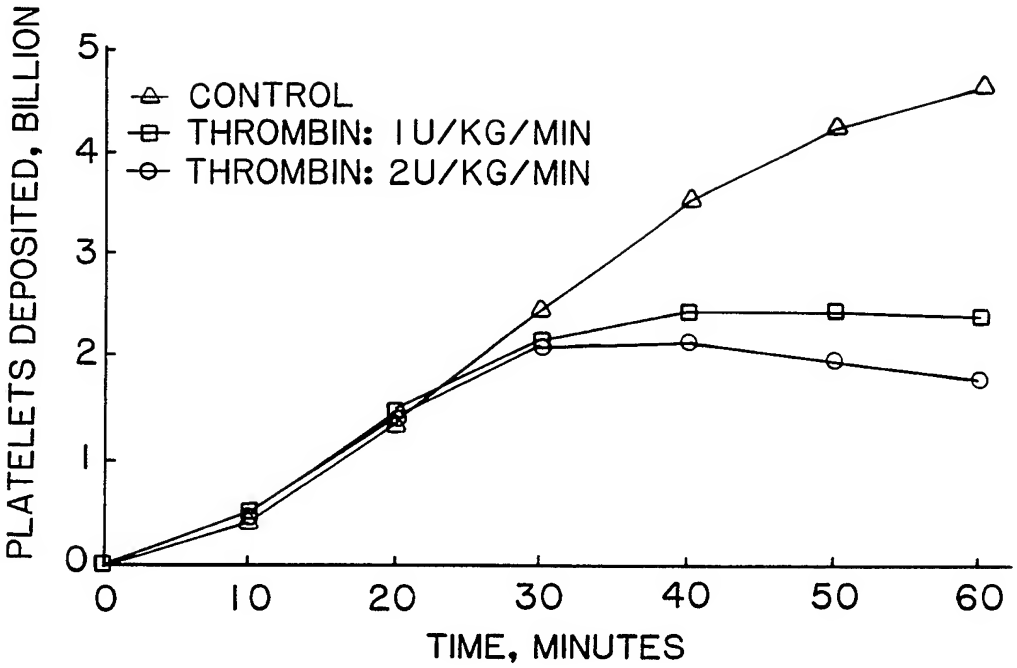
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1
2
3
4
5
CONTROL
THROMBIN: 1U/KG/MIN
THROMBIN: 2U/KG/MIN
0
10
20
30
40
50
60
TIME, MINUTES

FIG. 3

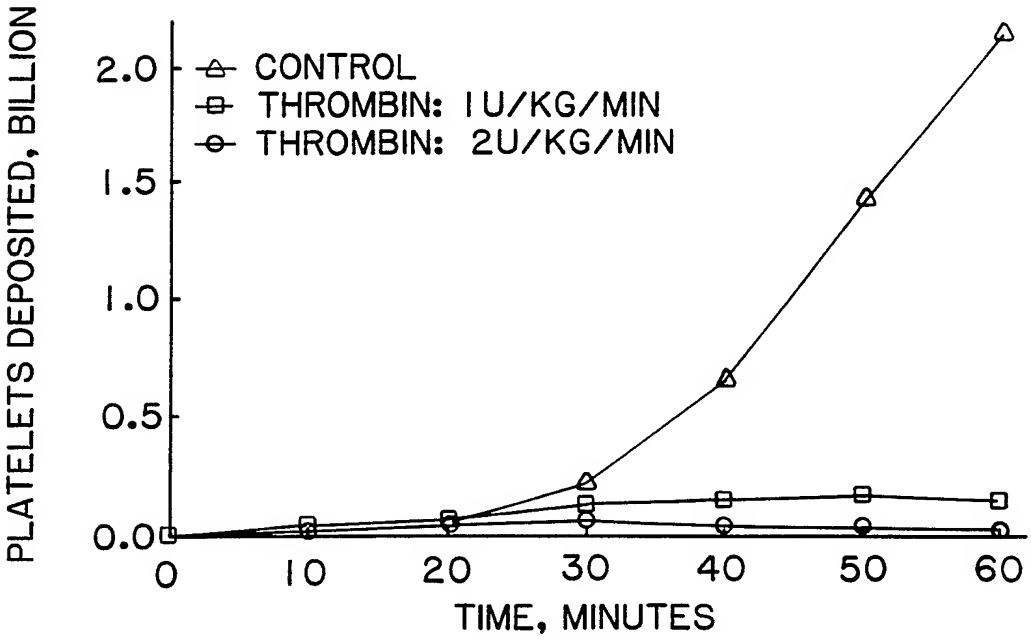
PLATELETS DEPOSITED, BILLION
0.0
0.5
1.0
1.5
2.0
CONTROL
THROMBIN: 1U/KG/MIN
THROMBIN: 2U/KG/MIN
0
10
20
30
40
50
60
TIME, MINUTES

FIG. 4

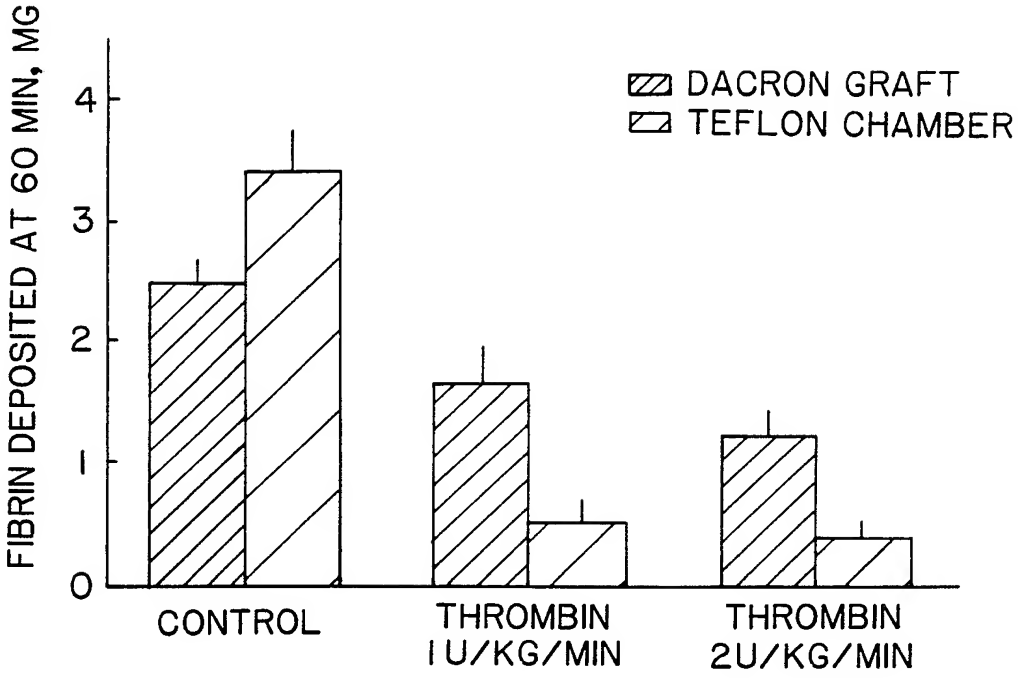
FIBRIN DEPOSITED AT 60 MIN, MG
0
1
2
3
4
DACRON GRAFT
TEFLON CHAMBER
CONTROL
THROMBIN 1U/KG/MIN
THROMBIN 2U/KG/MIN

FIG. 5

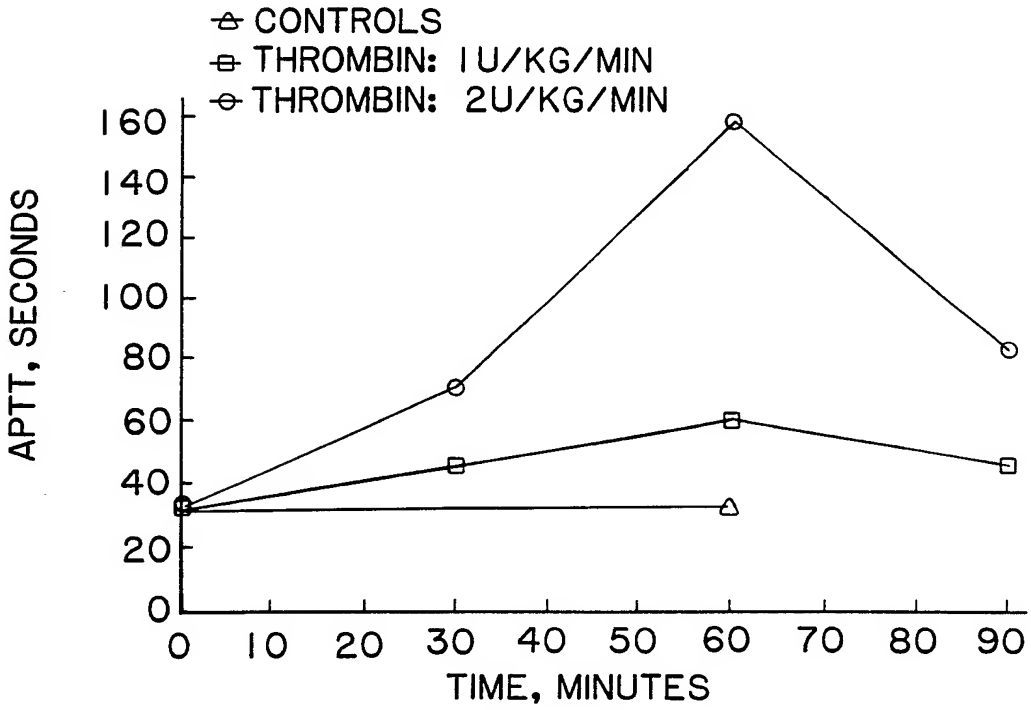
CONTROLS
THROMBIN: 1U/KG/MIN
THROMBIN: 2U/KG/MIN
APTT, SECONDS
0
20
40
60
80
100
120
140
160
0
10
20
30
40
50
60
70
80
90
TIME, MINUTES

FIG. 6

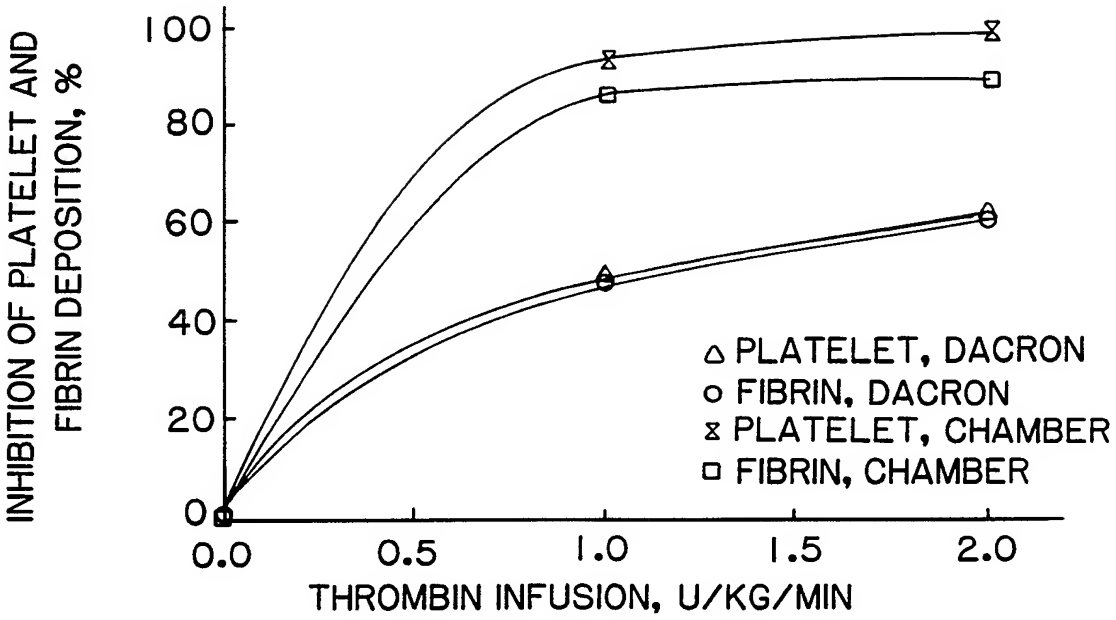
INHIBITION OF PLATELET AND FIBRIN DEPOSITION, %
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
THROMBIN INFUSION, U/KG/MIN
△ PLATELET, DACRON
○ FIBRIN, DACRON
⧖ PLATELET, CHAMBER
□ FIBRIN, CHAMBER

FIG. 7

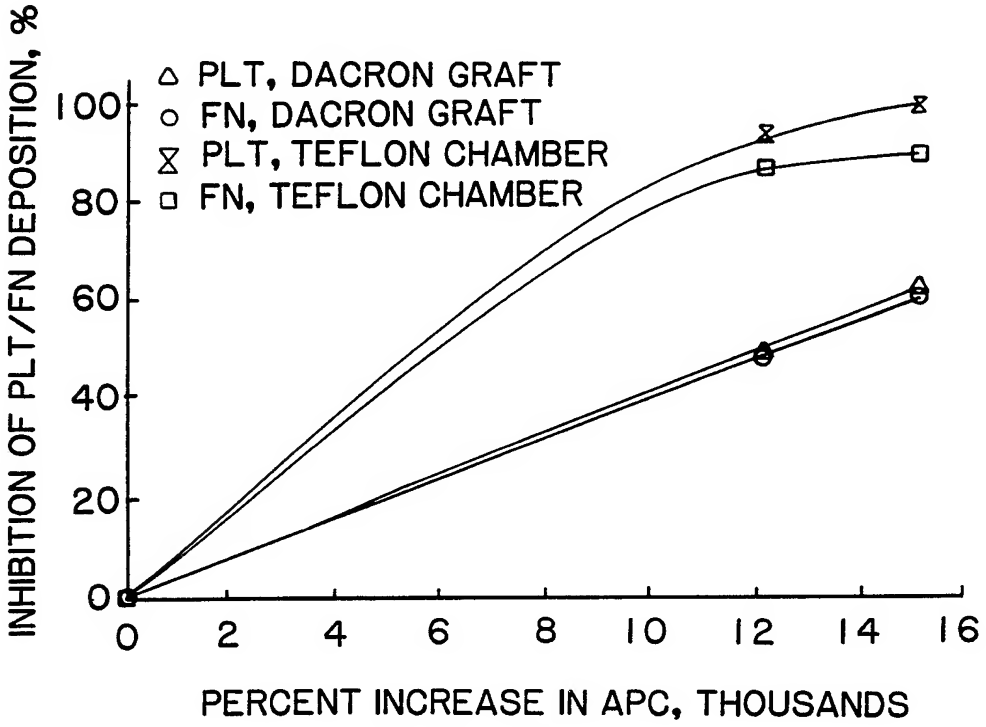
INHIBITION OF PLT/FN DEPOSITION, %
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
PERCENT INCREASE IN APC, THOUSANDS
△ PLT, DACRON GRAFT
○ FN, DACRON GRAFT
⧖ PLT, TEFLON CHAMBER
□ FN, TEFLON CHAMBER

FIG. 8

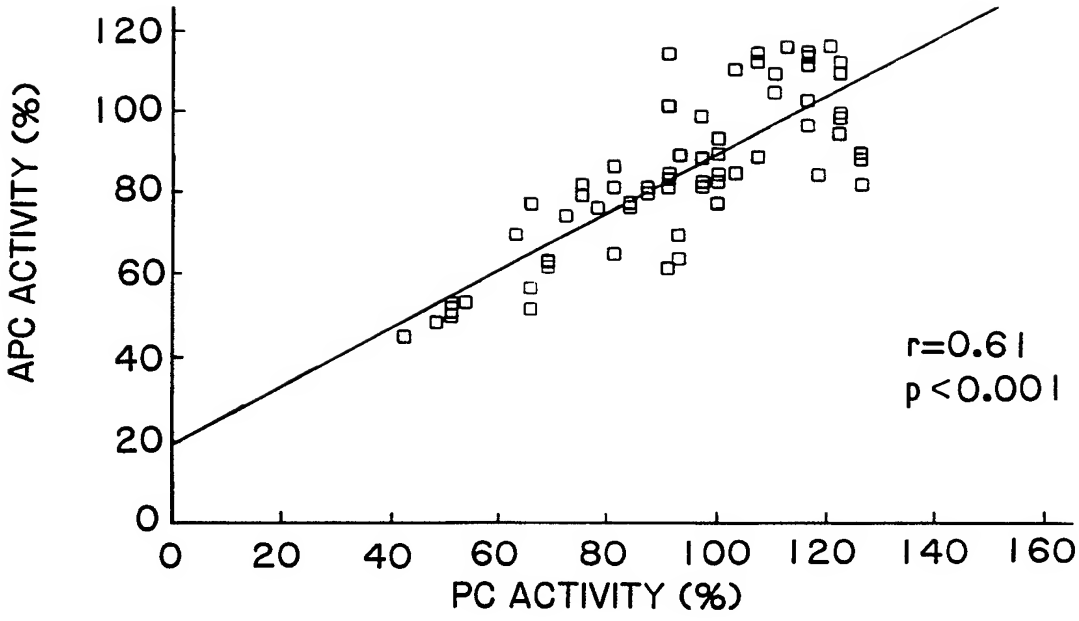
APC ACTIVITY (%)
120
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160
PC ACTIVITY (%)
r=0.61
p<0.001

FIG. 9

# INTERN. IONAL SEARCH REPORT

International application No.
PCT/US92/09978

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) : A[illegible]/547, 37/553; A61F 2/06; A61M 31/00; G01N 31/00
US CL : [illegible]; 623/1; 604/52; 436/15
According to [illegible]ional Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/94.64; 623/1; 604/52; 436/15

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

medline, biosis, embase, aps, ca, inpadoc

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | BIOCHEMISTRY, Vol. 78 (4), issued 1981, C.T. Esmon, et al., pages 2249-2252, especially page 2249. | 1-33 |
| X<br>Y | J. CLIN. INVEST., Vol. 70, issued July 1982, P. C. Comp, et al., pages 127-134, especially pages 127 and 132. | 1,5,10,12-17<br>1-33 |
| Y | THE EMBO JOURNAL, Vol. 9 (8), issued 1990, H. J. Ehrlich, et al., pages 2367-2373, especially pages 2367 and 2372. | 1-33 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be part of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

Date of the actual completion of the international search
15 March 1993

Date of mailing of the international search report
22 MAR 1993

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INTERNA .ONAL SEARCH REPORT

International application No.
PCT/US92/09978

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | [illegible] of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>Y | J. CLIN. INVEST., Vol. 74, issued July 1984, M. Colucci, et al., pages 200-204, especially page 200. | 1,5-6,10-16, 22-24<br>1-33 |
| Y | CHEMICAL AND PHARMACEUTICAL BULLETIN, Vol. 37 (3), issued March 1989, K. Hirahara, et al., pages 692-696, especially page 692. | 1-33 |
| X<br>Y | US, A, 4,849,403 (Stocker, et al.) 18 JULY 1989, Col. 8, lines 48-65<br>Col. 4-5, bridging paragraph. | 1, 11-12<br>1-33 |
| X,P<br>Y | US, A, 5,126,140 (Ito, et al.) 30 JUNE 1992, Col. 4-5, bridging paragraph<br>Col. 4-7. | 1,5-6,10-16, 22-23<br>1-33 |
| Y | US, A, 5,009,889 (Taylor, Jr., et al.) 23 APRIL 1991, Col. 1, lines 13-31, lines 44-49. | 1-33 |
| Y,P | US, A, 5,143,901 (Schwarz, et al.) 01 SEPTEMBER 1992, Col. 5, lines 66-67. | 1-33 |
| T | NATURE, Vol. 360, issued 19 November 1992, M. A. Richardson, et al., pages 261-264. | 1-33 |
| A | FED. PROC., Vol. 45 (4), #5339, issued 1986, M. D. Burdick, et al., page 1072. | 1-33 |
| A | THROMBOSIS RESEARCH, Vol. 37, issued 1985, F. B. Taylor, Jr., et al., pages 155-164. | 1-33 |
| A | J. CLIN. INVEST., Vol. 68 (1), issued November 1981, P. C. Comp, et al., pages 1221-1228. | 1-33 |
| A | THROMBOSIS AND HAEMOSTASIS, Vol. 63 (1), issued 1990, K. Okajima, et al., pages 48-53. | 1-33 |
| A | CIRCULATION, Vol. 84 (6), issued 1991, A. Gruber, et al., pages 2454-2462. | 1-33 |
| A,P | US, A, 5,084,274 (Griffin, et al.) 28 JANUARY 1992. | 1-33 |
| A,P | CARDIOLOGY, Vol. 80 (3-4), issued 1992, S. Goto, et al., pages 252-256. | 1-33 |